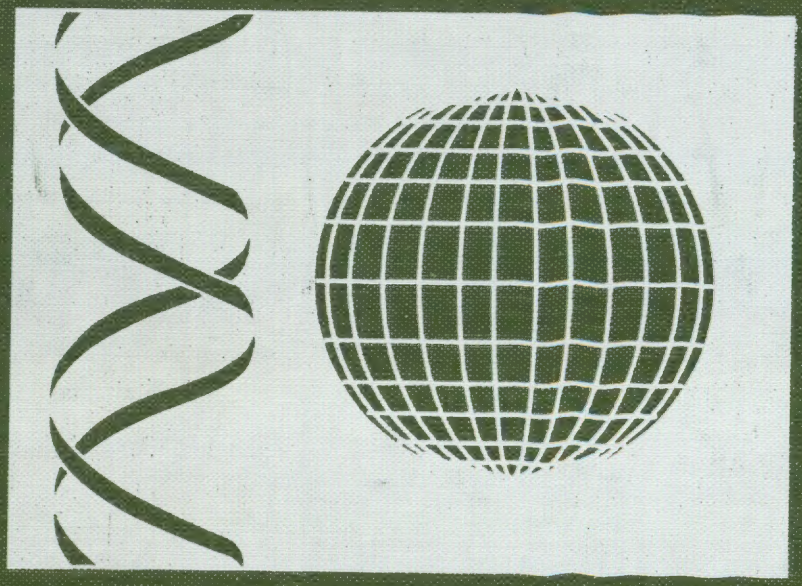
Conservation of Plant Genes II:

Utilization of Ancient and Modern DNA



Edited by
R. P. Adams
J. S. Miller
E. M. Golenberg
J. E. Adams

Missouri
Botanical
Garden

Conservation of Plant Genes II:

Utilization of Ancient and Modern DNA

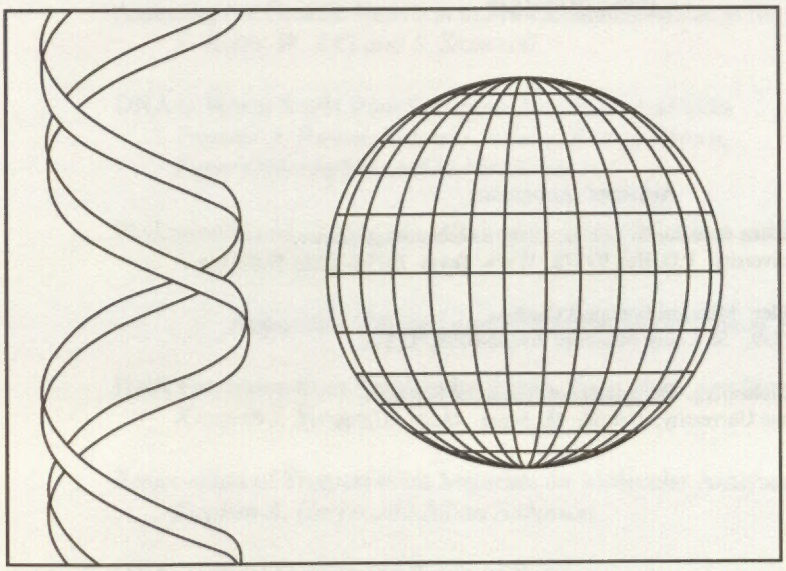
Edited by R. P. Adams, J. S. Miller, E. M. Golenberg & J. E. Adams

Missouri Botanical Garden

REF
QK1
.M833
1994
v.48
c.3

Conservation of Plant Genes II:

Utilization of Ancient and Modern DNA



Edited by
R. P. Adams
J. S. Miller
E. M. Golenberg
J. E. Adams

Missouri Botanical Garden

MISSOURI BOTANICAL
GARDEN LIBRARY

MONOGRAPHS IN SYSTEMATIC BOTANY

FROM THE MISSOURI BOTANICAL GARDEN

Volume 48

ISBN 0-915279-20-7
ISSN 0161-1542

AUTHORS' ADDRESSES

Robert P. Adams & Janice E. Adams: Plant Biotechnology Center, Baylor University, P.O. Box 97372, Waco, Texas 76798-7372, U.S.A.

James S. Miller: Missouri Botanical Garden, P.O. Box 299, St. Louis, Missouri 63166-0299, U.S.A.

Edward M. Golenberg: Department of Biological Sciences, Wayne State University, Detroit, Michigan 48202, U.S.A.

SERIES EDITOR
Marshall R. Crosby

MANAGING EDITOR
Amy Scheuler McPherson

EDITORIAL ASSISTANT
Diana Gunter

PUBLICATIONS STAFF
Magdalen Lampe
Robin Bruce

Copyright © 1994 by Missouri Botanical Garden.
All rights reserved.

CONTENTS

Preface vii

DNA Bank-Net - An Overview
Robert P. Adams 1

The Utilization of DNA from Ancient and Fossil Sources

Uncertainty in the Analysis of Fossil DNA Sequences
Edward M. Golenberg 15

Assessing the Genetic Variation in Pre-Columbian Maize at the Molecular Level
F. Rollo, W. Asci and S. Sassaroli 27

DNA in Wheat Seeds from European Archaeological Sites
Terence A. Brown, Robin G. Allaby, Keri A. Brown, Kerry O'Donoghue and Robert Sallares 37

Phylogenetic and Taxonomic Information from Herbarium and Mummified DNA
Scott O. Rogers 47

Extraction, Storage and Utilization of DNA from Extant Sources

DNA Extraction from Recalcitrant Plants: Long, Pure, and Simple?
Kenneth J. Sytsma 69

Preservation of Tropical Plant Materials for Molecular Analyses
Stephen A. Harris and Julian Robinson 83

DNA Archival Storage and Retrieval Systems
Elliott P. Dawson, Kan Wang, Shu-Ping Jiao, James R. Harris and James R. Hudson 93

Recent Advances in the Use of PCR Based Technology for DNA Fingerprinting in Plants
Stephen Smith 101

Polymerase Chain Reaction with Universal Primers (UP PCR) and its Applications to Plant Genome Analysis
S. A. Bulat, N. V. Mironenko, M. N. Lapteva and P. P. Strelchenko 113

The Use of RAPDs to Determine Germplasm Collection Strategies in the African Species, *Phytolacca dodecandra* (Phytolaccaceae)
Tigst Demeke and Robert P. Adams 131

RAPD Fingerprinting Cultivars of a Native Fruit Species (*Amelanchier alnifolia* Nutt., Rosaceae) A New Canadian Horticultural Crop
Brian J. Weir, Ravindra N. Chibbar and Richard G. St. Pierre 141

Camptothecin, an Anti-Cancer Alkaloid from *Camptotheca acuminata* (Nyssaceae)
Thomas D. McKnight and David D. Henning 149

Traditional and Modern Methods of Plant Conservation
Daniel K. Abbiw 159

DNA Bank Depositories in Developing Countries: The Way Forward in Sub Saharan Africa
A. A. Oteng-Yeboah 175

Nigerian Medicinal Plants and Danger of Extinction
Zac O. Gbile 187

Production of Abnormal Somatic Embryos and their Transferring into Normal Seedlings in Cotton Tissue Culture
Zheng Sijun 205

Intellectual Property Rights and Gene Utilization

Decision Points for Transferring Plant Intellectual Property to the Private Sector
Steven C. Price and Leanna M. Lamola 211

Natural Product Drug Discovery and Development at the National Cancer Institute: Policies for International Collaboration and Compensation
Gordon M. Cragg, Michael R. Boyd, Michael R. Grever, Thomas D. Mays, David J. Newman and Saul A. Schepartz 221

Biotechnology in Developing Countries: Participation by Private Industry
Robert B. Horsch 233

Models for Ethical Collaboration in Biodiversity Prospecting
James S. Miller and Daniel K. Harder 239

Bioresource-Based Opportunities for Scientific and Industrial Growth in Developing Countries
Kodzo Gbewonyo 245

Panel Discussion on Intellectual Property Rights and Gene Utilization 255

Commentary on Missouri Botanical Garden Symposium Discussion
Michael A. Gollin 273

PREFACE

The Missouri Botanical Garden is honored to have hosted the second meeting of the DNA Bank-Net from April 5 to 7, 1993. The Garden, which is the oldest in the United States, opened to the public in 1859. Currently the Garden's programs include over 50 Ph.D. scientists studying plants throughout Latin America, Africa, China, the former Soviet Union, North America, and tropical Asia. We base our research efforts in plant sciences in developing countries, where we are committed to strengthening botanical institutions and to providing opportunities for study and career development of individuals in those countries.

We recognize explicitly the fact that the developing countries of the world, in addition to having 77% of the world's population (a rapidly growing proportion), also have 80% of terrestrial biodiversity. Yet these same countries have only 6% of the world's scientists, and most of those are concentrated in China, India, Mexico, and Brazil, just a very few of the developing countries. For most developing countries, scientific talent is stretched so thin as to make it virtually impossible to understand organisms and natural communities, or to manage them for the national benefit. If it is impossible for a country to make decisions for its own benefit as to how to manage its own biodiversity, and in our case we are particularly interested in plants, then it is irrational to expect that country to be able to make agreements with other countries about matters of intellectual property rights, commercialization, or any of the other subjects of these meetings. The internal dependable advice that would make a government feel secure in making those agreements and the internal strength to be able to derive commercial benefit from the biodiversity is clearly inadequate in the developing world where the majority of biodiversity exists.

No one will protect, use, understand, and appreciate their own biodiversity more than an informed public. In that sense we are very interested in Instituto Nacional de Biodiversidad or INBio in Costa Rica and in the biodiversity institutes that are being created in Mexico and Taiwan. There are also growing efforts to create analogous institutes in Kenya and Indonesia, and to some extent in India and other countries. This emerging network of biodiversity institutes in developing countries holds great promise for building up the kind of capacity necessary to provide governments with the advice they need to make intelligent decisions about their biodiversity. This can make possible the kinds of developments that were the subject of the meeting, regarding collection and holding of DNA samples, intellectual property rights, commercialization, and other related matters. But, again, without appropriate institutional support, the situation will become quite impossible.

The Garden is particularly pleased with its ongoing relationship with the National Cancer Institute and its efforts to strengthen the appreciation, study, and management of biodiversity in various countries in Africa and in Madagascar, where we are committed to a long-term program of exploration, inventory, and conservation of the biota, and to development of the indigenous scientific community and institutions. We intend to remain committed to that indefinitely, because we look at it as the only really serious way to ensure plants are known, managed properly, appreciated, and studied scientifically.

The Garden is also very active in Russia and the other successor states of the former Soviet Union. Although we count the 350 million people who live in those states among the 1.2 billion or 23% of the people in the world, who comprise the industrialized countries, they are now suffering such a severe crisis that, unless we can help them to maintain their capabilities and activity in the field of plant biodiversity, they will soon be essentially a developing country. If we remain constant and help them to keep on understanding, studying, and managing information about their own plants effectively, they will be able to contribute to this worldwide effort.

On a larger scale, we are obviously living in a time when, owing to the combination of over-consumption among the world's industrialized countries, which have a shrinking 23% of the global population, and very rapid population growth in developing countries, the world's natural resources are being destroyed with dangerous speed. We engage in a national debate in the United States about the remaining 10% of ancient forests that we have in the Pacific Northwest, without fully realizing that the forests are essential to the appearance and character of Oregon and Washington; we reduce the discussion merely to the issue of preserving jobs. This means cutting down the trees in the next three years so that people can still be working in the sawmills for three more years, instead of taking seriously the need to reemploy them now. It is willful ignorance that could make us caricature this matter as a misguided effort to save the northern spotted owl, with the whole matter of regional values and the importance of forests and soils and climate and everything else involved in them being lost somewhere in the mix. That same kind of misguided attitude, derived from economic principles that unfortunately have developed in the Western world over the last 300 years, is also reducing resources throughout the world so rapidly that we are in danger of losing some 50,000 of the 250,000 estimated species of plants in the world over the next 30 years. If a visitor were to come to the world from another planet, that visitor would immediately conclude that we were insane, because we base our whole livelihood directly or indirectly on about a quarter of a million species of plants, and we don't pay any attention to them at all. How can it conceivably be, for example, that the United States can talk about spending 40 or 60 billion dollars on a space platform, and not be willing to spend 50 million dollars to preserve the 50,000 species of plants that are going right down the drain, and which the discussions at this meeting show very clearly are the primary source of medicines, foods, and antifungicides, in the whole world. It is absolutely incomprehensible behavior and it can be based only on a "me-first" attitude that we surely do need to overcome.

The subjects contemplated and discussed during the DNA Bank-Net meetings are very important parts of the strategy that must be applied during this difficult period of time, but nothing in that strategy is more important than international understanding or a caring for one another. This is true both inside the United States, where we engage to an unacceptable degree in discrimination against the poor and elderly, in sexism, and in racism, but even more so throughout the world, where industrialized countries, who have arrogated to themselves such an enormous proportion of the world's resources, refuse to look squarely at the world situation and understand that our futures are all interwoven with one another.

In a technical sense, this conference is a step toward laying secure plans for dealing with such matters as gathering DNA and other kinds of samples from wild plants, and intellectual property rights, which are kind of a shorthand for some of the deeper

problems that I have referred to briefly. We take the greatest pleasure in having hosted this meeting and commend both the seriousness of the issues and the importance of the deliberations about them. We applaud the efforts of the DNA Bank-Net and congratulate Bob Adams for having gotten a lot of this started.

5 April 1993
Peter H. Raven
Director, Missouri Botanical Garden

DNA BANK-NET - AN OVERVIEW

Robert P. Adams
Plant Biotechnology Center, Baylor University, BU Box 97372
Waco, Texas 76798, USA

SUMMARY

An overview of DNA Bank-Net is presented, including the rationale for the initiation and development. DNA Bank-Net will initially function as a reserve of DNA from rare and endangered tropical species and thus a genetic insurance policy against the loss of genes in the world gene pool. In a few years, DNA Bank-Net will begin dispensing genes via PCR (Polymerase Chain Reaction) using primers supplied by users.

INTRODUCTION

The modern movie "The Medicine Man" has sensitized the public concerning the number of novel insecticides, biocides, medicines, etc. that could exist in nature. Yet, the principal areas of diversity among plants, the lowland tropical forests, will have been cut or severely damaged within the next 20 years (Raven, 1987, 1988). The Amazon River system, for example, contains eight times as many species as the Mississippi River system (Shulman, 1986). Raven (1987, 1988) estimated that as many as 1.2 million species would become extinct in the next twenty years. The loss of plant species will mean a loss of potential plant derived pharmaceuticals, now estimated at $2 billion/year in the United States alone (U. S. Congress, 1987).

The first plant to plant gene transfer occurred in 1983 (Murai et al., 1983), and now genes have been transferred to plants from viruses (Nelson et al., 1988), bacteria (Barton et al., 1987; Della-Cioppa et al., 1987; Fischhoff et al., 1987) and even from mammals to plants (Lefebvre et al., 1987; Maiti et al., 1988). Genetic transfers are being performed in order to attain insect, bacteria, viral and fungal resistance, a more nutritionally balanced protein, more efficient photosynthesis, nitrogen fixation, and salt and heavy metal tolerance, to name a few. These kinds of gene transfers from one unrelated organism to another indicate that we must now view the world's genetic resources (genes, DNA) from a horizontal perspective in which gene transfers will cut across species, genera and family boundaries.

Vigna unquiculata (L.) Walp., discovered in a market in Ilorin, Nigeria, contains a protein that inhibits trypsin digestion by insects (Redden et al., 1984). This gene has been moved to tobacco (*Nicotiana*) where the trypsin-inhibiting gene is expressed and offers tobacco the same resistance against insects as in cowpea (Newmark, 1987). It is interesting to note that although a very active form of the gene has been found in a Nigerian cowpea, scarcely 100 of the world's 13,000 legume species have been examined for this gene. Yet, the tropical legumes, one of the most promising groups for the evolution of natural insecticides, will certainly be subject to considerable germplasm loss in the next decade.

Cultivated crops are extremely inbred for factors such as yield, uniform flowering and height, and cosmetic features of the products. This narrow genetic base has resulted in several disastrous crop failures. Ireland's potato (*Solanum tuberosum* L.) famine of 1846, which resulted in famine and the emigration of a quarter of its population, was due

to the fact (Plucknett et al., 1987) that their potatoes had no resistance to the late blight fungus (*Phytophthora infestans*). This can be traced to the lack of genetic diversity in Irish potatoes, which had been multiplied using clonal materials from just two separate South American introductions to Spain in 1570 and to England in 1590 (Hawkes, 1979).

A more recent example is the southern corn leaf blight (*Helminthosporium maydis*) in 1970 in the United States. Because almost all of the corn (*Zea mays* L.) in the United States was of hybrid origin and contained the Texas cytoplasmic male sterile line, our fields of corn presented an unlimited extremely narrow gene base habitat for the fungus. By the late summer, 1970, plant breeders were scouring corn germplasm collections in Argentina, Hungary, Yugoslavia and the United States for resistant sources (Plucknett et al., 1987). Nurseries and seed fields were used in Hawaii, Florida, the Caribbean, and Central and South America to incorporate the resistance into hybrid corn in time for planting in the spring of 1971 (Ullstrup, 1972). Without these genetic resources this technological feat would not have been possible.

Millions of dollars are being invested by the National Cancer Institute (NCI) in plant drug prospecting in a massive plant collecting effort in the tropics to find anti-cancer and anti-AIDS virus compounds (Booth, 1987). The plant collectors are gathering leaves and/or bark and air-dry the material for shipment to Maryland where it will be extracted and assayed against 100 cancer cell lines and the AIDS virus. Yet, **no genetic resources will be collected**! When a promising compound is found, the plants will have to be recollected. For extensive testing (as well as commercial utilization), plantations will have to be established in the tropics to provide material.

The base collections of plant specimens have been utilized for the formulation of our understanding of morphological variation among taxa. Indeed, without the great herbaria of the world, our knowledge of plant evolution would be fragmented at the least. As we have moved into the era of utilizing chemical data for systematic and evolutionary studies, methods of preserving plant materials for future (chemical) work have been largely ignored. We are usually content to file a voucher specimen to document our chemical studies. With the present level of support for plant collections, it is unlikely that much of the world's plant species can be preserved by freezing so that scientists might have access to the study of secondary compounds, enzymes, or DNA/RNA in the coming centuries.

FORMATION OF DNA BANK-NET

Concurrent with the advancements in gene cloning and transfer, has been the development of technology for the removal and analyses of DNA. DNAs from the nucleus, mitochondrion, and chloroplast are now routinely extracted and immobilized onto nitro-cellulose sheets where the DNA can be probed with numerous cloned genes. Recent advances in the technology for the extraction and immobilization of DNA, coupled with the prospect of the loss of significant plant genetic resources throughout the world, has led to the establishment of DNA Bank-Net, an international network of DNA repositories for the storage of genomic DNA on every continent.

A group of 18 scientists held the organizational meeting at the Royal Botanic Gardens, Kew, London, April, 1991 to share country and institutional experiences using *in vitro* biotechnology and particularly cryostorage of DNA and DNA-rich materials (Adams and Adams, 1992). Relatively few scientists were interested in a 'genetic insurance policy' when the idea of banking genomic DNA from plants was first proposed

(Adams, 1988, 1990). However, currently there are 40 institutions (representing 25 nations and every continent, see Appendix I) that have expressed interest in DNA Bank-Net (Figure 1).

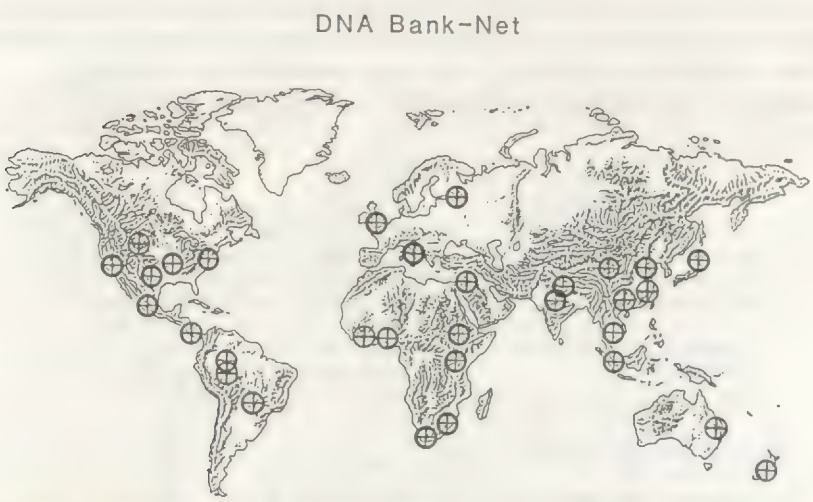


Figure 1. Map of individuals/institutions currently interested in DNA Bank-Net.

The conserved DNA will have numerous uses: molecular phylogenetics and systematics of extant and extinct taxa; production of previously characterized secondary compounds in trans-genic cell cultures; production of trans-genic plants using genes from gene families; *in vitro* expression and study of enzyme structure and function; and genomic probes for research laboratories.

STRUCTURE AND OPERATION OF DNA BANK-NET

At the organizational meeting of DNA Bank-Net, a task force was convened to define the functions of working (DNA dispensing) and reserve (base) nodes in the DNA Bank network. The group recommended the following functions (Adams and Adams, 1992):

Working (DNA dispensing) nodes:

- **a**. Collection of plant material by taxonomists. This may be the primary function of a particular node or be in association with other organizations such as universities, botanic gardens, etc.
- **b**. DNA extraction by molecular biologists or trained staff.
- **c**. Long-term preservation of DNA-rich materials and/or extracted DNA in liquid nitrogen.
- **d.** DNA analysis/gene replication by molecular biologists or trained staff.
- **e.** Distribution of DNA (genes, gene segments, oligonucleotides, etc.).

Reserve (base) nodes:

- **a**. Long-term DNA preservation in liquid nitrogen and monitoring of potential DNA degradation.
- **b**. Act as genetic reserve buffer for working nodes.

c. Replenishment of DNA if a working node experiences the catastrophic loss of storage parameters and DNA.

Figure 2 depicts the relationship between working and reserve nodes. Note the projected flow of plant materials and DNA through the working (DNA dispensing) node. It is likely that some of the working nodes would be actively acquiring and/or dispensing DNA from some geographic area (e.g., Africa), yet maintain separate cryovats, functioning as a reserve (base) node for another area (e.g., South America).

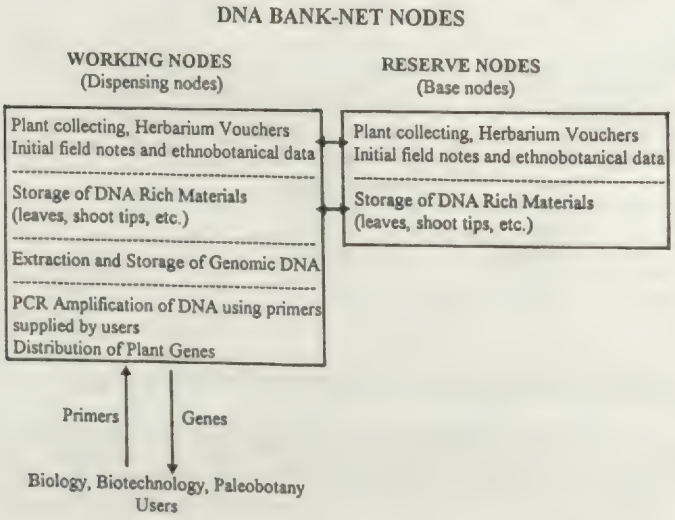


Figure 2. Schematic representation of the flow of materials and the relationship between working (DNA dispensing), reserve (base) nodes and users.

GENERAL REQUIREMENTS FOR NODES IN THE DNA BANK-NET

The task group recommended (Adams and Adams, 1992) that the following were the minimum requirements for nodes:

Working (DNA dispensing) nodes:
Personnel: Taxonomists/collectors, biochemists/molecular biologists, technicians for practical work, capable administration.
Equipment: Storage facilities (liquid nitrogen, cryovats), extraction facilities (centrifuges, gel electrophoresis, UV spectrophotometer, etc.), DNA Analyses and PCR duplication (PCR thermal cycler, micro-centrifuges, etc.), distribution system (packaging and mailing supplies), computer (database for inventory and correspondence).

Reserve (base) nodes:
Personnel: Technicians, capable administration.
Equipment: Storage facilities (liquid nitrogen, cryovats), computer (database for inventory and correspondence).

Each DNA collection should be split initially into at least 2 or 3 portions. One sample (DNA-rich material or extracted DNA) should be stored at a working (DNA dispensing) node and another portion(s) be stored in at least 1 (one), but desirably 2

(two) back-up reserve (base) nodes. The reserve nodes should be in different countries and, if possible, on different continents to safeguard the DNA samples against various natural and man-made catastrophes. Figure 3 shows a method that may be used to

Figure 3. Hypothetical example of a triplicate collection in Colombia. The DNA-rich materials are placed in silica gel or drierite for interim preservation and taken to the working node (A, Medellin). From Medellin (A), two replicates would be sent to reserve nodes at (B) Vavilov Institute and (C) the Missouri Botanical Garden. This procedure would minimize the amount of paperwork needed in the field.

distribute DNA-rich materials to working and reserve nodes. Material would be taken back to Medellin (A) and then the replicates sent to the Vavilov Institute (B) and the Missouri Botanical Garden (C). The plant materials (in silica gel) could be stored in a freezer until the identification and field notes have been accomplished and then shipped in quantity with other samples in off-season periods.

An alternative method is that materials might be collected in triplicate by a Colombian expedition and one of the replicates mailed to the Vavilov Institute (where it would be cryostored as a reserve node), another replicate might be mailed to the Missouri Botanical Garden (where it would be cryostored as the second reserve). The third replicate would then be taken home to the working node. This method would aid in assuring that the samples actually get sent to the reserve nodes, but it would be critical that considerable identification and label making being done in the field. No doubt other strategies will be developed with experience.

Several general recommendations came from the task groups (Adams and Adams, 1992) and these include:

a. DNA should be extracted from cryo-preserved DNA-rich materials only when the DNA is needed. Delaying the extraction has the advantage of letting technology catch up, so advanced techniques can be used as they become available.
b. Working nodes should generally be an existing organization with adequate biochemical expertise and have an associated herbarium. Having an herbarium on

site would not be required but a very close, local (in the city) association with a recognized herbarium (Holmgren et al., 1990) is required.

c. For the working as well as reserve nodes, it is necessary to have a strong institutional commitment, not just a personal commitment, in order that the collection be maintained in perpetuity not just for the lifetime of one person who has committed himself to the idea.

d. Consideration should be made concerning the availability of dependable electricity and liquid nitrogen in determining the feasibility of establishing a node.

e. Considerable interest was shown in the concept of storing composite DNA samples (e.g., a composite of DNA from all the legumes in a region, to be used for screening or retrieval of unusual genes).

f. The need for computer and data base compatibility was expressed. Given the number of flat file and relational data bases that are compatible with dBASE, it would seem that dBASE compatibility would be desirable. No consensus was reached in regards to this nor on the use of a flat file vs. relational data base. It was felt that the critical issue at present was to begin collecting DNA-rich materials.

SCOPE OF PLANT COLLECTIONS

The task group given this assignment felt that there is a need for an initial focus rather than random collections and that economically useful plants should be given some priority (Adams and Adams, 1992). However, this priority would not include the major crop plants of commercial usage that are widely cultivated (e.g., maize, rice, wheat, etc.), but rather those indigenous species that are tended and/or otherwise used by local people.

One problem with giving a priority to species is that field collecting then becomes 'plant hunting' trips, which tend to be very expensive. It would seem that the cheapest and most practical way to preserve the largest percentage of plant genes would be to utilize the current (and additional) floristic collectors (such as those of the Missouri Botanical Garden, Royal Botanic Gardens, etc.), who are already in the field and familiar with the vegetation in the region. The collections of DNA-rich material (leaves) could be done with little additional effort when specimens are collected.

COLLECTING PROCEDURES

DNA collectors should be considered the same as all other plant collectors. Consequently they should (Adams and Adams, 1992):

a. Voucher all collections in recognized herbaria (i.e., listed in Index Herb. ed. 8).

b. Provide proper label information as to the locality, habitat, etc., for each plant collected.

c. Follow all procedures concerning permits, convenios, and deposition of duplicate vouchers in the country of origin.

d. Collect leaf samples and pack them in desiccants (see Adams et al., 1992) immediately (the same day). Leaves are of value as simple long-term storage.

e. In the case of legumes, samples of root nodules should be taken if possible, but kept as a separate accession.

f. If a chemical treatment is used in the field, information should be provided concerning the method and some untreated leaves must be stored in desiccant (see d. above).

g. Fossil plants - When possible, fossil material should be included in DNA Bank-Net. In this case, when destruction of the source material occurs, documentation via photographs and fragments is necessary.
h. Some material may be accessioned from herbarium specimens under control of local curators using current methods of DNA extraction. Herbarium sheets should be marked if sampled for DNA. Herbarium specimens are limited in supply and their utility appears to be limited to material collected without chemical preservation. Material may be sampled directly from the sheet or the attached specimen envelope if it contains sufficient leaf material (ca. 0.1 - 0.5 g dry wt.) for DNA extraction.

INTERIM FIELD STORAGE OF SPECIMENS

The problems associated with bringing back fresh or frozen materials can generally be overcome by specialists (e.g., world-wide collections of fresh foliage of *Juniperus* for essential oil analyses and DNA by RPA). However, botanists doing floristic research will likely collect many of the specimens from tropical rare and endangered species. They often collect specimens from scores of different species in a single day. The bulk of the materials that they have to process and ship requires that any protocol for the collection of samples for specialized needs (e.g., DNA storage / analyses), must be quick, simple and trouble-free. The generalist collector, working in tropical areas cannot be expected to preserve hundreds or thousands of collections for months under tropical conditions and then arrange transport through customs, all the while keeping the individual specimens frozen.

Fortunately, at least as far as DNA preservation is concerned, interim preservation in silica gel or drierite is an effective way to keep plant materials in the field and/or in transit for several months at ambient temperatures (Adams et al., 1992). Genomic DNA from fresh spinach stored for up to 6 months still contains high molecular weight DNA (see Adams et al., 1992 for detailed discussion).

PROTOCOL FOR FIELD PRESERVATION OF FOLIAGE

Drierite has a water capacity of 10 to 14 percent, but above 6.6%, the capacity varies inversely with temperature (W. A. Hammond Drierite Co.). One would not want to risk possible rehydration of leaves, so storage ratios should be based on the 6.6% capacity. In lab tests, silica gel absorbed 8.85% of its weight of water after exposure to 100% humidity for 16 h at 22°C. We have found that plant materials contain as much as 92% moisture, so a useful approximation would be to assume the plant is mostly water and use 16 to 20 times the fresh leaf weight for the drierite or silica gel component.

Now that inexpensive ($100 USD) battery powered, portable balances are available, one could take a supply of jars that hold (for example) 100 g of silica gel and then weigh out 5 g of fresh leaf material and add it to the jar along with silica gel (or drierite). We have found that air dried leaves (suitable for herbarium vouchers) generally contain from 10 to 15% water. Using a robust value of 20% water for air dried leaves, one can weigh out 5 g of air dried leaves (5 g x 20% = 1 g water) or 1 g fresh leaves per 20 g of silica gel. This procedure may seem time consuming, but in practice, we merely do a quick check on the leaf area needed to give approximately 1 g (fresh leaves) or 5 g (dried leaves) and then just use that amount of leaf area. For example, for spinach, a 2 cm x 4 cm fresh leaf area weighs about 1 g. So, one can just cut the leaves into roughly 2 cm x 4 cm squares and add one square to 20 g of silica gel. For succulent leaves, a

slightly different protocol may be used. Liston et al. (1990) removed succulent leaf material after 24 hours in drierite and placed it in fresh drierite. A note of caution is necessary concerning field drying of specimens for subsequent silica gel/ drierite storage. We have experienced difficulty obtaining DNA from leaves dried at temperatures higher than about 55°C. In very rainy conditions where high drying temperatures (from butane stoves, for example) are used to dry specimens, it would seem advisable to merely blot leaves free of surface moisture and then place the fresh leaf material directly into silica gel or drierite. Liston et al. (1990) took 2-5 g of plant tissue and wrapped it in tissue paper to prevent it from fragmenting, then placed it in a 125 ml Nalgene bottle, 1/3 prefilled with drierite (with blue indicator crystals), and then filled the bottle (2/3) with additional drierite.

Plastic bottles are probably to be preferred to glass, to avoid breakage in transit. Using clear jars allows one to check the indicating crystals without opening the jar. The lids should be sealed with vinyl tape to insure against moisture leakage. The use of parafilm to seal containers is not recommended, as we have found it to come loose at 37° C (and of course, at tropical temperatures!).

Silica gel and drierite do differ in one characteristic that may be a consideration. We have found that silica gel can be dried (recharged) at 100°C for 24 h but drierite must be dried at much a higher temperature (200°C). In addition, we could easily dry silica gel in a microwave oven, but were unable to dry (recharge) drierite in a microwave oven. If the desiccant gets wet before use, silica gel appears to be much easier to dry. Silica gel is used in large quantities for flower drying and, thus, may be cheaper, depending on the source. Both drierite and silica gel could be recharged for reuse on subsequent trips, but one should be very careful to remove any leaf fragments. If the materials are to be checked through customs, it is useful to have a small container of silica gel/ drierite that you can open and show the customs agents. A demonstration that the blue indicator crystals will turn pink when you breath on or moisten them is helpful in convincing the customs officials **not** to open your sealed specimen jars.

FUTURE RESEARCH

The vast resources of dried herbarium specimens may hold considerable DNA that would be suitable for PCR. It seems likely that the integrity of DNA would decrease with the age of specimens. Because there are many types of herbaria storage, preservation and collections, there is a need for systematic investigations of the effect of modes of preparation, collection and storage on the integrity of DNA in the world's major holdings.

One of the major concerns in storing DNA from extinct species is the limited amount of DNA available for distribution. A general process by which the DNA could be immobilized and then specific genes or oligonucleotides amplified is needed. Figure 4 depicts the immobilization of genomic DNA onto nylon as described by Kadokami and Lewis (1990) for cDNA from spiders (*Nephila clavipes*). Amplification would then involve removing the membrane with the bound DNA from cryo-storage (Figure 5) and amplifying the desired gene, washing away the primers and placing the bound DNA back into cryostorage. Although Kadokami and Lewis (1990) reported successful PCR amplification of membrane bound cDNA, we have not been able to extend their work to genomic plant DNA. Additional research is needed in this area.

Research is needed to amplify the entire genome DNA of a species. Some modification of the GAWTS (Genomic Amplification with Transcript Sequencing,

Sommer et al., 1990) type protocol needs to be developed for eventual supplementation of DNA reserve stocks and obviate the need for replenishment from outside sources.

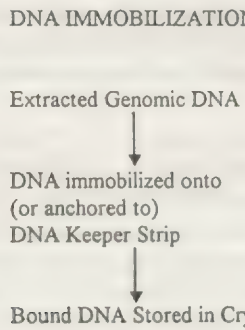


Figure 4. Diagram for the immobilization of genomic DNA (after Kadokami and Lewis, 1990 and Adams and Adams, 1992).

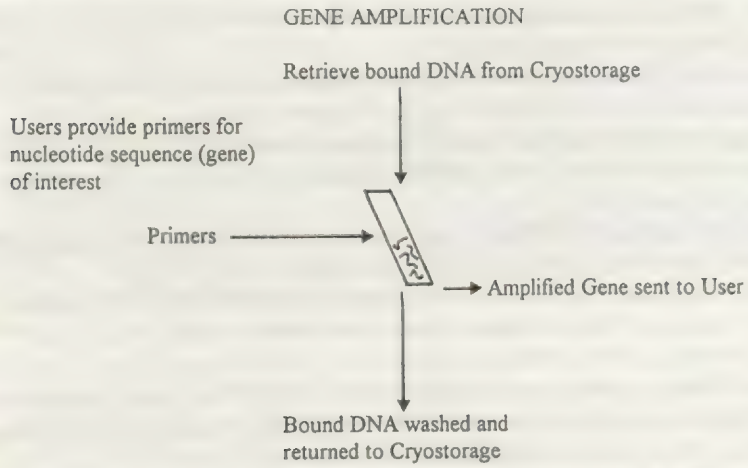


Figure 5. Scheme for the amplification of immobilized genomic DNA (after Kadokami and Lewis, 1990 and Adams and Adams, 1992).

CONCLUSIONS

DNA Bank-Net is an association that fills a needs for a professional organization that would function initially as a lead organization and superstructure. The association brings together capabilities and expertise, produces a newsletter, and coordinates DNA banking activities.

Technical workshops need to be conducted in order to bring experts together and develop specific techniques and protocols for DNA extraction, amplification and storage. DNA Bank-Net supports the concept of using license agreements and/or contracts that insure that there is a flow of money back to the countries and/or institutions when commercialization of protected germplasm is achieved. This will guarantee the uninhibited exchange of germplasm for scientific purposes, so that scientific achievements are not obstructed.

DNA Bank-Net should complement activities already being performed by different institutions, specifically, those working in the area of germplasm collection and conservation.

Technically, all the necessary expertise is available to begin the collection and storage of DNA from endangered species. Now, the vision is needed.

ACKNOWLEDGEMENTS

DNA Bank-Net has been supported by funds from Baylor Univerity (President's Fund), the Conservation, Food and Health Foundation, the Helen Jones Foundation and the Wallace Genetic Foundation. A special thanks to Dr. Herbert H. Reynolds, President, Baylor University, for making funds available for support of the second DNA Bank-Net meeting in St. Louis.

REFERENCES

Adams, R.P. 1988. The preservation of genomic DNA: DNA Bank-Net, AIBS Meeting, Aug., Univ. of California, Davis, CA.

Adams, R.P. 1990. The preservation of Chihuahuan plant genomes through *in vitro* biotechnology: DNA Bank-Net, a genetic insurance policy, *in*: "Third Symposium on Resources of the Chihuahuan Desert Region," A.M. Powell, R.R. Hollander, J.C. Barlow, W.B. McGillivray and D.J. Schmidly, eds., Printech Press, Lubbock, TX.

Adams, R.P. and Adams, J.E. 1992. "Conservation of Plant Genes: DNA Banking and *in vitro* Biotechnology," Academic Press, NY.

Adams, R.P., Do, N. and Chu, G-L. 1992. Preservation of DNA in plant specimens by dessication, *in*: "Conservation of Plant Genes: DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, NY.

Barton, K.A., Whiteley, H.R. and Yang, N. 1987. *Bacillis thuringiensis* -endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to Lepidopteran insects. Plant Physiol. 85:1103-1109.

Booth, W. 1987. Combing the earth for cures to cancer, AIDS. Science 237:969-970.

Della-Cioppa, G., Bauer, S.C., Taylor, M.L., Rochester, D.E., Klein, B.K., Shah, D.M., Fraley, R.T. and Kishore, G.M. 1987. Targeting a herbicide resistant enzyme from *Escherichia coli* to chloroplasts of higher plants. BioTechnology 5:578-584.

Fischhoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, D.E., Rogers, S.G. and Fraley, R.T. 1987. Insect tolerant transgenic tomato plants. BioTechnology 5:807-813.

Hawkes, J.G. 1979. Genetic poverty of the potato in Europe, *in*: "Proceeding of the Conference: Broadening the Genetic Base of Crops," A.C. Zeven and A.M. van Harten, eds., Wageningen, Netherlands, 3-7 July, 1978. PUDOC, Wageningen.

Holmgren, P.K., Holmgren, N.H. and Barnett, L.C. 1990. "Index herbariorum. Part I. The herbaria of the world," ed. 8., Regnum Veg. 120.

Kadokami, Y. and Lewis, R.V. 1990. Membrane bound PCR. Nucleic Acid Res. 18: 3082.

Lefebvre, D.D., Miki, B.L. and Laiberte, J-F. 1987. Mammalian metallothionein functions in plants. BioTechnology 5:1053-1056.

Liston, A., Rieseberg, L.H., Adams, R.P., Do, N. and Zhu, G-L. 1990. A method for collecting dried plant specimens for DNA and isozymes analyses, and the results of a field test in Xinjiang, China. Ann. Mo. Bot. Gard. 77:859-863.

Maiti, I.B., Hunt, A.G. and Wagner, G.J. 1988. Seed-transmissable expression of mammalian metallothionein in transgenic tobacco. Biochem. & Biophys. Res. Comm. 150:640-647.

Murai, N., Sutton, D.W., Murray, M.G., Slightom, J.L., Merlo, D.J., Reichert, N.A., Sengupta-Gopalan, C., Stock, S.A., Barker, R.J., Kemp, J.D. and Hall, T.C. 1983. Phaseolin gene from bean is expressed after transfer to sunflower via tumor inducing plasmid vectors. Science 222:476-482.

Nelson, R.S., McCormick, S.M., Delannay, X., Dube, P., Layton, J., Anderson, E.J., Kaniewska, M., Proksch, R.K., Horsch, R.B., Rogers, S.G., Farley, R.T. and Beachy, R.N. 1988. Virus tolerance, plant growth, and field performance of transgenic tomato plants expressing coat protein from tobacco mosaic virus. BioTechnology 6:403-409.

Newmark, P. 1987. Trypsin inhibitor confers pest resistance. BioTechnology 5:426.

Plucknett, D.L., Smith, N.J.H., William, J.T. and Murthi Anishetty, N. 1987. "Gene Banks and The World's Food," Princeton University Press, Princeton, New Jersey.

Raven, P.H. 1987. Forests, people, and global sustainability, Keynote Address, National Audubon Society Biennial Convention, Western Washington University, Bellingham, WA.

Raven, P. H. 1988. Tropical floristics tomorrow. Taxon 37:549-560.

Redden, R.J., Singh, S.R. and Luckefahr, M.J. 1984. Breeding for cowpea resistance to Bruchids at IITA. Protection Ecol. 7:291-303.

Shulman, S. 1986. Seeds of controversy. BioScience 36:647-651.

Sommer, S.S., Sarkar, G., Koeberl, D.D., Bottema, C.D.K., Buerstedde, J-M., Schowalter, D.B. and Cassady, J.D. 1990. Direct sequencing with the aid of phage promoters, *in*: "PCR Protocols," M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds., Academic Press, San Diego.

Ullstrup, A.J. 1972. The impacts of the southern corn leaf blight epidemics of 1970-1971. 1 Annual Review of Phytopathology 10:37-50.

U.S. Congress 1987. "Technologies to maintain biological diversity," Office of Technology Assessment, Washington, D.C.

Appendix I. Individuals/institutions that have expressed an interest in DNA Bank-Net.

Dr. Robert P. Adams. DNA Bank-Net Director, Plant Biotechnology Center
P.O. Box 97372, Baylor University, Waco, TX 76798

Dr. Daniel K. Abbiw, Botany Department, University of Ghana
Box 55, Legon, Ghana, West Africa

Dr. Vishwanath P. Agrawal, Research Laboratory for Agricultural Biotechnology and Bichemistry, Kathmandu, Nepal

Drs. Lucia Atehortua/Ricardo Callejas, HUA, Department of Biology
University of Antiquia, Medellin, Colombia

Dr. Luiz Antonio Barreto de Castro, CENARGEN/EMBRAPA
Parque Rural, CP 102372, W 70770, Brasilia DF, Brazil

Dr. Mike Bennett, Director, Jodrell Labs, Royal Botanic Gardens
Kew, Richmond, Surrey, TW9 3AB, England

Dr. Brian M. Boom, Vice President for Botanical Science
The New York Botanical Garden, Bronx, NY 10458-5126

Dr. Anthony H.D. Brown, Division of Plant Industry, CSIRO
GPO Box 1600, Canberra, ACT 2601, Australia

Prof. Cheng Xiongqying , Institute of Nuclear Agricultural Sciences
Zhejiang Agricultural University, Hangzhou, 310029 China PRC

Dr. Ram Chaudhary, Research Lab. for Agricultural, Biotechnology & Biochemistry
P.O. Box 2128, Kathmandu, Nepal

Dr. Mark Chase, Jodrell Labs, Royal Botanic Gardens, Kew,
Richmond, Surrey, TW9 3AB, England

Dr. Thomas Elias, Director, Rancho Santa Ana Botanic Garden
1500 North College Ave., Claremont, CA 91711

Prof. J. Eloff, Director, National Botanical Institute
Private Bag X101, Pretoria, 0001 South Africa

Dr. Z. O. Gbile, Director,Forestry Research Institute of Nigeria
Private Mail Bag 5054, Ibaden, Nigeria

Dr. Chaia C. Heyn, Dept. of Botany
The Hebrew University, Jerusalem, 91904, Israel

Dr. Vernon Heywood, IUCN Conservation Monitoring Center
53 The Green, Kew, Richmond, Surrey, TW9 3AA, England

Dr. Toby Hodgkin, Research Officer,IBPGR, c/o FAO of the UN
Via delle Sette Chiese 142, 00145 Rome, Italy

Prof. Hu Zhong, Kunming Institute of Botany,
The Academy of Sciences of China, Heilongtan, Kunming, Yunnan, China PRC

Dr. Kunio Iwatsuki, Botanical Gardens, University of Tokyo
3-7-1 Hakusan, Bunkyo-ku ,Tokyo 112, Japan

Dr. S. L. Kapoor, Cytogenetics Lab, National Botanical Research Institute
Rana Pratap Marg., Lucknow 226 001, India

Prof. Mupinganayi Kadiakuida, Director General, CARI
B.P. 16513, Kinshasa, Republic of Zaire

Prof. Lin Zhong-ping, Division of Plant Molecular Biology
Institute of Botany, Academia Sinica, Beijing 100044, China PRC

Dr. Ma Cheng, Chief Engineer, Chinese Academy of Sciences (Academia Sinica)
52 San Li He Road, Beijing, China PRC

Appendix I (contd.)

Dr. Lydia Makhubu, University of Swaziland
Kwaluseni Campus P/Bag, Kwaluseni, Swaziland

Dr. John S. Mattick, Centre for Molecular Biology and Biotechnology
University of Queensland, St. Lucia, Queensland, QLD 4072, Australia

Dr. James Miller, Missouri Botanical Garden
2345 Tower Grove Ave., St. Louis, MO 63110

Dr. Titus K. Mukiama, Department of Botany, University of Nairobi
Chiromo, P.O. Box 30197, Nairobi, Kenya

Dr. Wickneswari Ratnam, Forestry Research Institute, Karung Berkunci 201, JLN FRI
Kepong, 52109, Kuala Lumpur, Malayasia

Dr. Alfred Apau Oteng-Yeboah, Dept. of Botany, University of Ghana
P.O. Box 55, Legon, Ghana

Dr. Bart Panis, Laboratory of Tropical Crop Husbandry, Catholic University of Leuven
Kardinaal Mercierlaan 92, B-3001 Heverlee, Belgium

Dr. Ghillean T. Prance, Director, Royal Botanic Gardens
Kew, Richmond, Surrey, TW9 3AB, England

Dr. Steve Price, Office of Intellectual Property
Iowa State University, Ames, IA 50010

Dr. Vishwanath P. Agrawal, Research Lab for Agric. Biotechnology
and Biochemistry, P.O. Box 2128, Kathmandu, Nepal

Dr. Loren Rieseberg, Rancho Santa Ana Botanic Garden
1500 North College Ave., Claremont, CA 91711

Dr. W. Roca, Head, Bio-Tech Unit
CIAT AA6713, Cali, Colombia

Dr. Phillip Stanwood, National Seed Storage Lab, USDA
Colorado State University, Ft. Collins, CO 80523

Dr. Dennis Stevenson, The New York Botanical Garden
Bronx, NY 10458-5126

Dr. Peter Strelchenko, N. I. Vavilov Institute of Plant Industry
42, Herzen Street, 190000, St. Petersburg, Russia

Dr. Panie Temiesak, Head, Seed Technology, Central Lab & Greenhouse Complex
Kasetsart University, Nakorn Pathom 73140, Thailand

Dr. Victor M. Villalobos, FAO/AGP, Via delle Terme di Caracalla
0010 Rome, Italy

Prof. Luz Ma. Villarreal de Puga, Instituto de Botanica University de Guadalajara
Apartado 139, Las Agujas Nextipac, Zapopan, Jalisco, Mexico

Dr. Melaku Worede, Plant Genetic Resources Center
P.O. Box 30726, Addis Ababa, Ethiopia

Prof. Zheng Sijun, Dept. of Agronomy, Zhejiang Agricultural University
Hangzhou, 310029, Zhejiang, China PRC

Prof. Zhu Ge-lin, Institute of Botany, Northwest Normal University
Lanzhou, Gansu, China PRC 730070

UNCERTAINTY IN THE ANALYSIS OF FOSSIL DNA SEQUENCES

Edward M. Golenberg
Department of Biological Sciences, Wayne State University
Detroit, Michigan 48202, USA

SUMMARY

The utilization of fossil and subfossil DNA for studies of molecular evolution holds great promise especially in light of the successful recovery of endogenous DNA from plant and animal fossils of Miocene age and from specimens preserved under a wide variety of conditions. Yet, in spite of these results, progress has been slow and frustrating. To be sure, much of the lack of success is due to technical problems concerning the extraction and purification of the ancient DNA and to logistic problems concerning the availability of appropriate fossil material. However, additional procedural and theoretical limitations may also be contributing to our slow progress. Improper formulation of criteria for verification may increase the probability of rejection of true fossil sequences (Type II errors). In particular, dependence on phylogenetic inferences for authentication will result in mistaken rejection of true fossil sequences when the fossils themselves have been mis-identified, when post depositional modification of DNA augments branch lengths possibly leading to changes in tree topology, or when phylogenetic algorithms do not infer the true tree under conditions of unequal branch lengths and short gene sequences. Additionally, simulated data indicate that nucleotide substitution rate estimates may be influenced by the topology of the true tree. If the fossil is a direct ancestor of an extant specimen, the sample variance will be inversely proportional to the age of the fossil. If the fossil is not a direct ancestor, the sample variance will be directly proportional to the age of the last shared common ancestor. When fossil branch lengths are long, that is when the fossil is young and the bifurcation with the modern specimen is old, more substitutions may accumulate by chance on the fossil branch than on the longer (in time) extant branch leading to negative rates of nucleotide substitution.

INTRODUCTION

Research in plant fossil DNA analysis has been proceeding in several laboratories over the past three to four years. Yet with the exceptions of the publication of two primary DNA sequences from the Miocene Clarkia Deposits (Golenberg et al., 1990; Soltis et al., 1992), and few additional unpublished sequences, only slow progress has been made. An only slightly improved record has been established with fossil and subfossil animal DNA. Here, too, recent reports (Cano et al., 1992; DeSalle et al., 1992) have demonstrated that DNA can survive in Miocene deposits. Together, the Clarkia plant material and the older Dominican insects in amber establish that DNA may be preserved in a wider range of conditions (e.g., Janczewski et al., 1992) and for much longer periods of time than has been thought (Pääbo and Wilson, 1991; Sidow et al., 1991). Given this growing acknowledgement of the potential for long term DNA preservation and the obvious utility of the evolutionary analysis of fossil gene sequences, why has this area developed at such a slow pace? Certainly one reason is the lack of availability of suitable material, much of which can only be collected during appropriate field seasons. However, a number of other reasons for this slow progress are due to

technical (Golenberg, 1991, 1993) and procedural problems that are particular to fossil and subfossil DNA research. In this paper, I will highlight some of these procedural problems with examples and suggest, where possible, alternative approaches to compensate.

AUTHENTICATION OF FOSSIL AND SUBFOSSIL SAMPLES

Higuchi et al. (1984) published the first DNA sequence from an extinct species, the quagga. They analyzed the amino acid data derived from the nucleotide sequences using parsimony methods to establish the relatedness of the cloned quagga DNA to the extant zebra. Golenberg et al. (1990) explicitly used two criteria for authentication of a Miocene *Magnolia* sequence. First, the sequence must prove to be unique, and, therefore, not be a contaminant, and second, the sequence must group with sequences from purportedly related species under phylogenetic analysis. Similar criteria have been used in subfossil DNA papers (e.g., Cooper et al., 1992; Hagelberg and Clegg, 1991; Lawlor et al., 1991).

The first problem that arises with this approach is a philosophical problem. The first test, that the experimental sequence is not a contaminant, has been approached in a number of ways. When dealing with fossil material, we have framed the question in terms of the uniqueness of the sequence. This makes intuitive sense for two reasons. First, it is likely that the fossil will not be a direct ancestor to a modern specimen, and, therefore, there must be some period of time in which the fossil or the modern sequences will acquire independent nucleotide substitutions. Second, the modern specimen will at least have the period of time since the deposition of the fossil in which to acquire independent substitutions. (I will address both of these issues in more detail below.) We, therefore, set up our null hypothesis that the fossil sequence is different from all other sequences. Of course, this means that our alternative hypothesis is that the sequence is identical to another sequence. This design, however, is inherently incorrect and leads to untestable experiments and to insupportable conclusions. A null hypothesis, in our case that our fossil sequence is different from all other sequences, is never directly supported, but only rejected. In comparing sequences, we are ultimately limited to the data that are available at a given point in time. The test then can never be legitimately used to authenticate a fossil sequence. If we do not reject the null hypothesis in our initial tests, we must continually test our results as new data are published. This obviously is not done, but more importantly, it is not necessary.

The purpose of this first criterion is not to establish uniqueness, but rather to test against contamination. Students of subfossil or ancient DNA have been sensitive to this problem precisely because the experimental sequences may be identical to modern sequences. A several hundred year old maize or human sequence will very likely be identical to a modern sequence. Therefore, non-uniqueness is not a legitimate basis for rejection of authenticity of a sample. As an alternative approach many of these workers have identified potential sources of contamination, and tested experimental sequences against sequences directly derived from these sources. The null hypothesis is that the experimental sequence is identical to one of an established set of sequences. This hypothesis is testable, and we may readily identify false positives resulting from contamination. As the most common sources of contamination of plant DNA are plant DNA's that are present in the laboratory or previously amplified PCR products, each laboratory will establish different sets of test sequences.

The second problem arises from the use of molecular phylogenetic inferences to establish the relation of our purported fossil sequence to sequences derived from modern species that are expected to be close relatives. This test is again often useful in identifying false positives when the results clearly contradict expectations. For example, if a DNA sample from a fossil that morphologically clearly belongs within the Taxodiaceae produces a sequence that groups with Poaceae sequences, then whether or not Poaceae DNA is present in the laboratory, one can assume that the sequence is a false positive and not an authentic fossil sequence. However, a number of conditions may result in an unexpected phylogenetic inference which may not be a true indication of a false positive. The conditions include 1.) mis-identification of the fossil, 2.) post depositional modification of the DNA, and 3.) incorrect phylogenies derived from the phylogenetic algorithms.

Mis-identification may either be systematic or occasional. A fossil may be identified as belonging to one family while actually belonging to another. An example of this is *Cocculus heteromorpha* from the Clarkia deposit. The genus *Cocculus* belongs to the Menispermaceae. Recently, Manchester et al. (1991) has reclassified these fossils as *Nordenskioldia* belonging to the Trochodendraceae based on reproductive structures. Clearly, legitimate fossil sequences from similarly mis-identified species may be rejected as false positives based on the unexpected phylogenetic affiliations. Similarly, a single leaf fossil may be mis-identified as a result of a hasty inspection in the field prior to on site processing of the fossil. Many species have similar leaf silhouettes. Naturally occurring individual and developmental variation and incomplete preservation may obscure interspecific differences in cursory identifications. Inconsistencies between expected phylogenetic affinities and observed inferred affinities may be the result of such identifications. The resolution of these inconsistencies can only come by reexamining both the field identification and the DNA sequence. It is clear that all collection and processing of compression fossils for DNA extractions must include the separate preservation of sample cuticles as vouchers. This may seem trivial to many systematists, but it has not been standard operating procedure up until now.

In the first report on ancient DNA, Higuchi et al. (1984) identified post depositional or post mortum modification of DNA as a potential source of sequence variation. They suggested that nucleotide substitutions were being added to their sequence which were not in the original animal DNA. They took the unexpectedly long fossil branch on the phylogenetic tree as an indication of such modified errors. We may include in this modification, errors in DNA polymerization inherent to the polymerase being used. Damaged template DNA may induce additional errors either by forcing read through at AP sites or jumping of incomplete strands from one template to another resulting in intermolecular recombination (Lawlor et al., 1991; Pääbo et al., 1990). Errors producing nucleotide site substitutions will artificially increase branch lengths in phylogenetic analyses and may result in incorrect tree topologies. Intermolecular recombination, especially when working with a nuclear heterozygotic gene, or, worse, when background contamination is present, may result in intractable data.

Systematists have rapidly embraced numerical methods for phylogenetic inferences, especially when using direct DNA sequence or restriction fragment polymorphism data. With a few exceptions, systematists interpret the phylogenetic trees produced by the various analytic approaches as hypotheses of the true tree. This is reflected by the attention given to estimation of branch length variances (Nei et al., 1985), frequency of support of bifurcations in bootstrapped samples (Felsenstein, 1985),

and tests of significance of differences among alternative trees (Hasegawa and Kishino, 1989). Additionally, all phylogenetic inference methods are based on a number of assumptions which may be violated in real data. The different methods differ in their sensitivity to such violations. Nei (1991) recently investigated the probabilities of obtaining the correct tree from simulated data under different analytical approaches. When branch lengths vary the probability of obtaining the correct tree are invariably low. In addition, small number of nucleotide sites also reduces the probability of obtaining the correct tree. For example, when analyzing 300 bp of a slowly evolving gene (U=0.05) where true branch lengths vary, the probability of obtaining the true tree was .30 or less depending on the methods used. Unfortunately, these are exactly the conditions under which we analyze fossil sequences. However, as opposed to regular molecular systematic analyses, fossil analyses do not approach inferred trees as hypotheses, but rather as tests of hypotheses. If the inferred trees do not correspond to our expectations of the phylogenetic position of the fossil, we reject the sequence as being of doubtful authenticity. Obviously if the inferred tree has a low probability of being correct, we will have a large type II error.

USE OF FOSSIL DNA SEQUENCES FOR ESTIMATION OF NUCLEOTIDE SUBSTITUTION RATES

Related to the problem of verification of fossil sequences by phylogenetic inferences are problems related to the usefulness of this sequence data for evolutionary questions. Previously, I have suggested that fossil sequences may be used to estimate rates of nucleotide substitutions (Golenberg, 1993). Estimation of rates of substitution from extant sequence data requires the knowledge of the time to the last shared common ancestor of the two species, a time which is usually not known with any certainty. As an alternative, estimating rates with fossil data does not require the knowledge of the time to the last shared ancestor, but only the time of fossil deposition. The differences of branch lengths to an outgroup between an extant species and a fossil relative may be divided by the age of the fossil to obtain the rate of substitution. A list of a number of rate estimates of either the chloroplast genes *rbc*L and *atp*B or of *rbc*L alone is given in Table 1. The first five estimates are use an estimated time to shared common ancestor and the last three estimates use time of deposition of the fossil. The estimates based on sequences from extant taxa range from 4.2×10^{-11} to 1.9×10^{-9} substitutions x site^{-1} x year^{-1}. The estimates based on fossil sequences give low to negative values.

The first question that must be asked is if this method gives legitimate estimates or if there are inherent biases. To address this question, I studied the behavior of the estimates under varying topologies in simulation. The first tree topology used is shown in Figure 1. Four extant species are on the tree related by two bifurcation events. Each bifurcation event is 50 time units apart (50 million years). Nucleotide substitutions are expected to occur along the branches following a Poisson distribution. The rate of substitutions is .001 per time unit. Treating each time unit as a million years, this would be comparable to a rate of 1×10^{-9} substitutions per site per year which is approximately a mean rate for the chloroplast gene *rbc*L. In the first simulation, the fossil species is assumed to be a direct ancestor of species 4. The age of the fossil varies, moving along the branch of Species 4. Each topology is replicated 100 times to produce sequences for the four terminal taxa and the fossil. The sequences were analyzed using the DNA dist program of PHYLIP to estimate pairwise distance. The distance matrices

Table 1 Estimated rates of nucleotide substitution (substitutions x $Site^{-1}$ x $Year^{-1}$ x 10^{-9})

Taxa	All Sites	3rd Codon Pos.	Reference
Monocot-Dicot (*rbc*L & *atp*B)		1.9	Zurawski & Clegg, 1987
Spinach-Tobacco (*rbc*L & *atp*B)		1.3	Zurwaski & Clegg, 1987
Barley-Maize (*rbc*L)	0.67	1.2	Zurwaski et al., 1984
Arecaceae (*rbc*L)	0.058-0.130 (restriction sites)	0.042 (direct sequence)	Wilson et al., 1990
Taxodium (*rbc*L)	0.42-0.49		Soltis et al., 1992
Magnolia (*rbc*L) (outgroup *Platanus*)	-0.12		This paper
Pseudofagus (*rbc*L) (outgroup *Casuarina*)	0.19		This paper
Taxodium (*rbc*L) (outgroup *Metasequoia*)	0.042		This paper

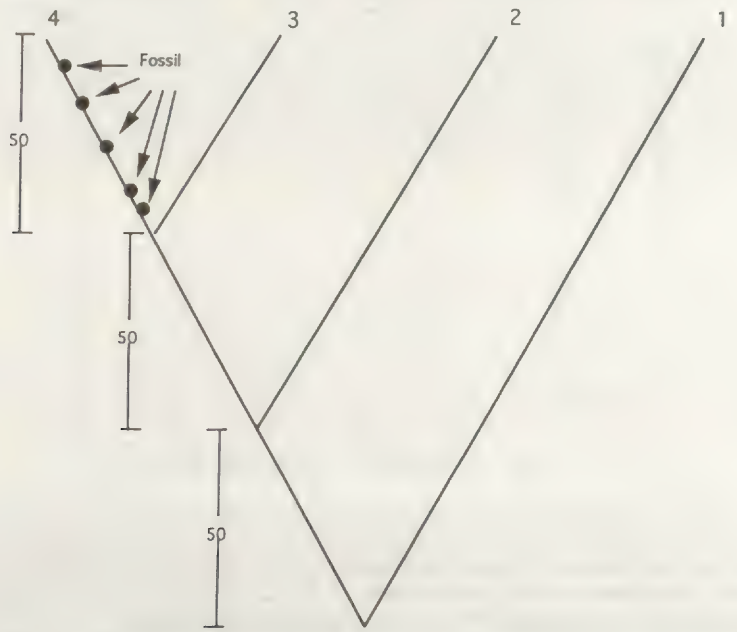


Figure 1. A phylogenetic tree used for simulating data. Species 1, 2, 3, and 4 are modern species, and are separated by three bifurcation events each occurring at intervals of 50 time units. A single fossil species is a direct ancestor of species 4. The age of the fossil species is varied from 45, 40, 30, 20, and 10 time units in separate simulations.

were analyzed using the Fitch-Margoliash algorithm (Fitch and Margoliash, 1967) (Fitch in PHYLIP) (Felsenstein, 1990).

The means of the 100 replicate estimates and their standard errors are plotted by the age of the fossil in Figure 2. The means were tested for significant differences from

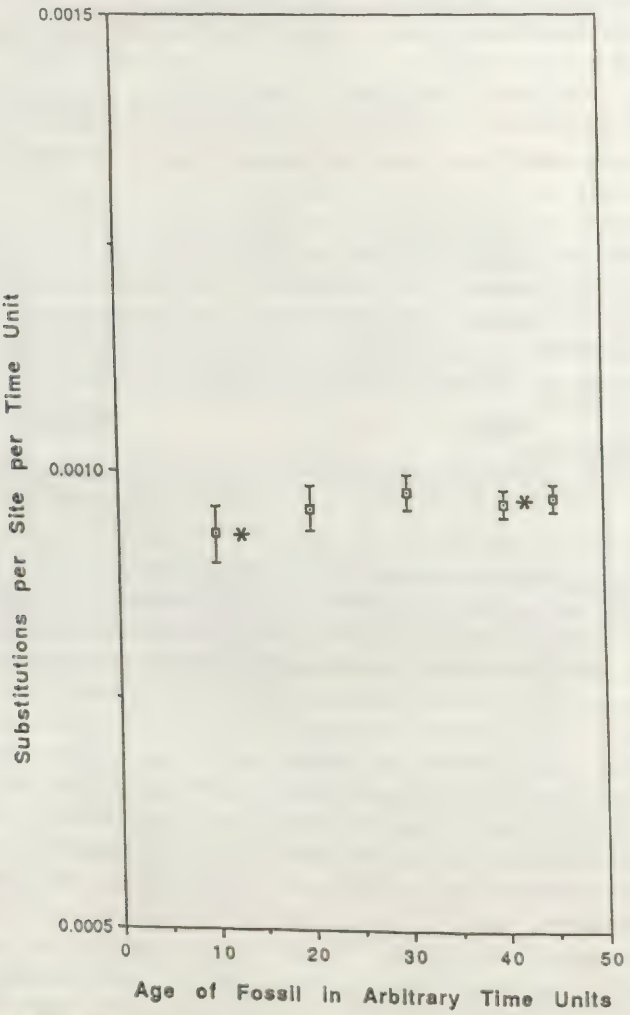


Figure 2. Plot of means of estimation of rates of nucleotide substitutions per site per time unit by the age of the fossil specimen. Fossils are direct ancestors of modern species. The age of the fossil represents the time before present in which the fossil sequence ceased to evolve. Vertical bars represent one standard error around the estimates. Asterisks indicate significant differences between mean estimates and the parametric value.

the parametric value of .001 using a t test. Differences in the variance were tested for significance using the F_{max} and Bartlett's test of homogeneity of variances (Sokal and Rohlf, 1981). Three results are apparent from these studies. 1.) The method may tend to underestimate the rates of substitution. It should be noted, however, that when the results of the second simulation are considered together with these results, there does not appear to be a consistent underestimate. 2.) Estimates of rates in fossil lines that are 10 and 40 time units old significantly differ from the true rate of 0.001. 3.) Sample variances differ significantly with the age of the fossil. Older fossils have smaller

variances. Both Bartlett's test and the F_{max} test indicate heteroscedasticity significant at a level of $p<0.001$.

While these results were not expected, they are consistent with increased variance due to small sample sizes. Because the fossil species is a direct ancestor, the only informative branch length is the branch since fossil deposition. The substitutions are accumulating along this branch following a Poisson distribution. Obviously, the longer the time period along a branch will be the smaller the effect of sampling. As a result, the older fossils give better estimates of true substitution rates and have smaller variances.

The Fitch-Margoliash algorithm estimated the true tree topology at frequencies of 0.91, 0.99, 1.00, 1.00 and 1.00 when the fossil was 45, 40, 30, 20, and 10 time units old, respectively. These results differ from those of Nei (1991), but the difference may be due to the smaller number of taxa involved in this study. The probability of inferring the correct tree seems to improve in those simulations where the estimates of substitution rates were poor.

In the second simulation, the rates and times of bifurcation of the four extant species are identical to the first simulation. The fossil species is now not a direct ancestor to species 4 but shares a common ancestor with species 4 before the common ancestor of species 3 (Figure 3). The age of the fossil (the time of fossil deposition) is

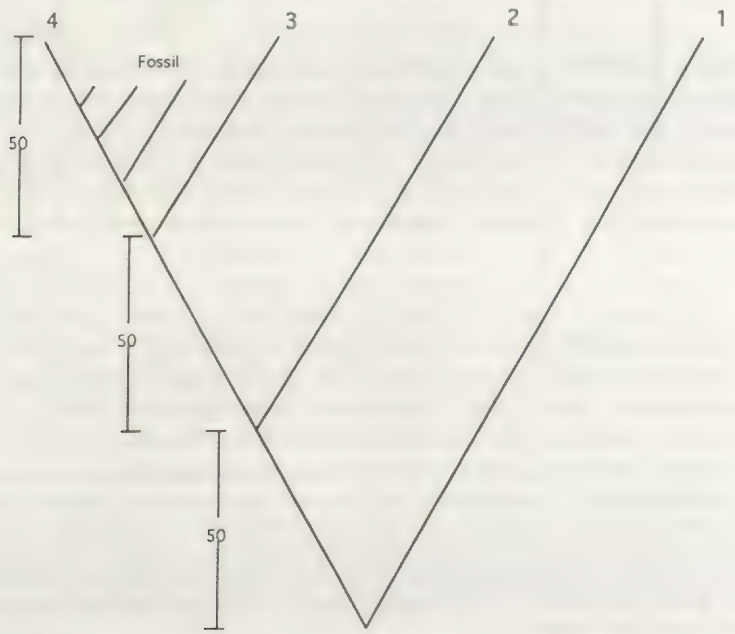


Figure 3. A phylogenetic tree used for simulating data. The phylogenetic relationships among the extant species are as in Figure 1. A single fossil species is not a direct ancestor of species 4 but shares a common ancestor with 4. The age of the fossil species is held constant but the time of divergence is varied from 45, 30, 20, and 10 time units before present in separate simulations.

held constant to 10 time units, however, the bifurcation point is varied along the branch from 45, 40, 30, 20, and 10 time units before present. I again used the PHYLIP programs DNADist and Fitch to analyze the simulated sequences (Felsenstein, 1990). The results are plotted in Figure 4.

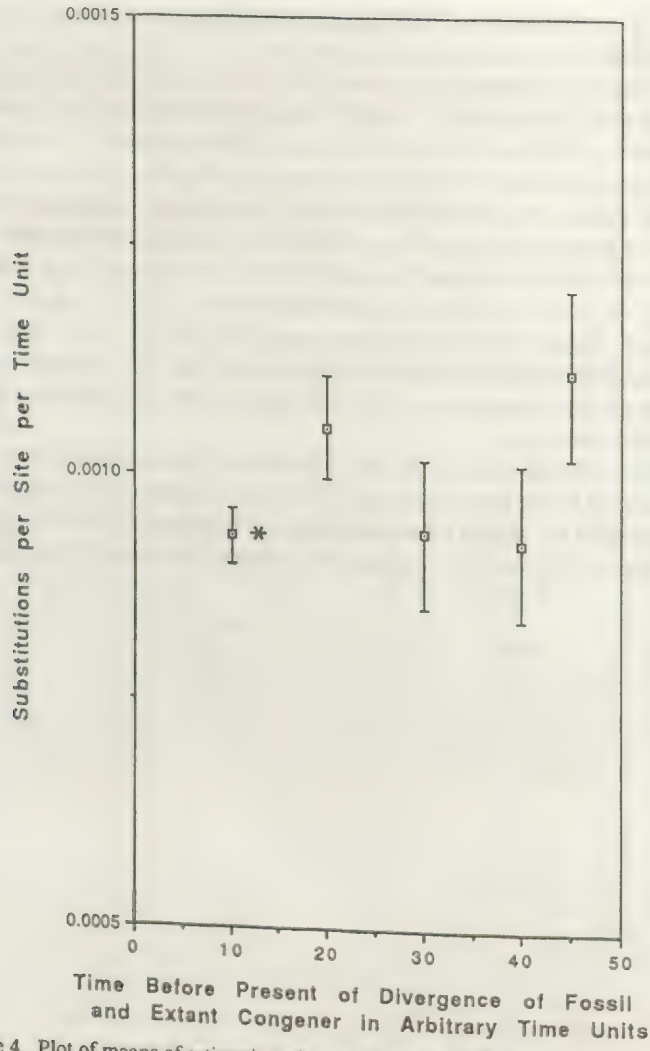


Figure 4. Plot of means of estimation of rates of nucleotide substitutions per site per time unit by the age of the fossil specimen. Fossils are not direct ancestors of modern species but have diverged from the closest modern relative at specified times before present. The ages of the fossils are held constant at ten time units before present and represent the time before present in which the fossil sequence ceased to evolve. Vertical bars represent one standard error around the estimates. Asterisks indicate significant differences between mean estimates and the parametric value.

The simulation at 10 time units before present is identical to the last study as the fossil has a zero branch length, and therefore the same data point was used. As in the previous study, the mean of the estimates is significantly different from 0.001. Differing from the previous study, none of the other estimates are significantly different from 0.001 even though the age of the fossil remains constant at 10 time units. The variances, as can be seen by the standard errors, increase greatly with the time to the last shared common ancestor, and differ significantly. When the shared common ancestor was at 45, 40, 30, and 20 time units old (compared to the 50 time unit bifurcation to species 3),

negative estimated rates of substitutions were found. This means that more substitutions occurred on the shorter time branch of the fossil than on the branch to the extant species even though the actual rates were identical. This implies that the topology of the true tree (which is never known) will influence the confidence limits of our estimates.

The probability of inferring the true tree varied even more than in the simulations with the direct ancestor. When the shared fossil/species 4 common ancestor was 10, 20, 30, 40 and 45 time units, the probabilities of inferring the correct tree were 0.80, 0.99, 1.00, 1.00 and 1.00. These initial results again suggest that there is an increased chance of estimating the true phylogenetic relationship when the length of the shared common branch of the fossil and the extant congener increases. This is as expected.

Lastly, distance matrices taken from the simulation of a 10 time unit old direct ancestor were analyzed by two different branch estimating algorithms, Fitch-Margoliash and Neighbor joining (Saitou and Nei, 1987). In this single test, the neighbor joining algorithm gave estimates that were not significantly different from the true rates, while using the same distance matrices, the Fitch Margoliash method gave a mean value that was significantly different. While this test does not show that the neighbor joining will be better under all conditions, it does demonstrate that different algorithms will give different results. This analysis is being expanded to test a wider range of conditions.

CONCLUSION

We can summarize the conclusions from these studies as follows. 1.) Rate estimates may be influenced by the true tree topologies and by the tree-generating algorithms utilized. 2.) Variances of rate estimates will also be influenced by the tree topologies. If the fossil is a direct ancestor of the extant species, variance is inversely proportional to the age of the fossil. Alternatively, if the fossil is not a direct ancestor of the extant species, the variance is directly proportional to the age of the last shared common ancestor. 3.) Phylogenetic inferences based on nucleotide sequence data often may not indicate the true tree, leading to Type II errors.

While having painted a rather pessimistic picture, I think it necessary to emphasize that many of the problems addressed here also apply to analyses of data from extant species. Some of these problems are addressed by molecular systematists, such as confidence tests of tree topologies, and some are not. The difference in approach when dealing with fossil data stems from the fact that we must rely more heavily on the conclusions of our tests. Clearly, while success rates in recovering fossil DNA still remains low, the potential remains great. We are making progress on overcoming some of the technical difficulties of fossil DNA amplification, and so I think that we can be optimistic that our success rate will improve with experience. I remain conservatively optimistic that we will reach some of the potential of fossil DNA research in the future.

REFERENCES

Cano, R. J., Poinar, H. and Poinar Jr., G. O. 1992. Isolation and partial characterization of DNA from the bee *Proplebeia dominicana* (Apidae: Hymenoptera) in 25-40 million year old amber. Med. Sci. Res. 20:249-251.

Cooper, A., Mourerchauvire, C., Chambers, G.K., Von Haeseler, A., Wilson, A.C. and Pääbo, S. 1992. Independent origins of New Zealand Moas and Kiwis. Proc. Natl. Acad. Sci., U.S.A. 89:8741-8744.

DeSalle, R., Gatesy, J., Wheeler, W. and Grimaldi, D. 1992. DNA sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. Science 257:1933-1936.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783-791.

Felsenstein, J. 1990. PHYLIP Manual Rel. 3.3, University Herbarium, University of California, Berkeley, California.

Fitch, W. M. and Margoliash, E. 1967. Construction of phylogenetic trees. Science 155:279-284.

Golenberg, E. M. 1991. Amplification and analysis of Miocene plant fossil DNA. Phil. Trans. R. Soc. Lond. B 333:419-427.

Golenberg, E. M. 1993. Fossil Samples, *in:* "Ancient DNA", B. Herrmann and S. Hummel, eds., Springer Verlag, New York.

Golenberg, E. M., Giannassi, D. E., Clegg, M. T., Smiley, C. J., Durbin, M., Henderson, D. and Zurawski, G. 1990. Chloroplast DNA sequence from a Miocene *Magnolia* species. Nature 344:656-658.

Hagelberg, E. and Clegg, J.B. 1991. Isolation and characterization of DNA from archaeological bone. Proc. R. Soc. London B. 244:45-50.

Hasegawa, M. and Kishino, H. 1989. Confidence limits on the maximum-likelihood estimate of the hominoid tree from mitochondrial-DNA sequences. Evolution 43:672-677.

Higuchi, R., Bowman, B., Friedberger, M., Ryder, O.A. and Wilson, A. C. 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312: 282-284.

Janczewski, D.N., Yuki, N., Gilbert, D.A., Jefferson, G.T. and O'Brien, S.J. 1992. Molecular phylogenetic inference from saber-toothed cat fossils of Rancho-La-Brea. Proc. Natl. Acad. Sci. U.S.A 89:9769-9773.

Lawlor, D. A., Dickel, C.D., Hauswirth, W.W. and Parham, P. 1991. Ancient *HLA* genes from 7,500-year-old archaeological remains. Nature 349:785-788.

Manchester, S. R., Crane, P.R.and Dilcher, D.L. 1991. *Nordenskioldia* and *Trochodendron* (Trochodendraceae) from the Miocene of northwestern North America. Bot. Gaz. 152:357-368.

Nei, M. 1991. Relative efficiencies of different tree-making methods for molecular data, *in:* "Phylogenetic Analysis of DNA Sequences," M. M. Miyamoto and J. Cracraft, eds., Oxford University Press, New York.

Nei, M., Stephens, J.C. and Saitou, N. 1985. Methods for computing the standard errors of branching points in an evolutionary tree and their application to molecular data from humans and apes. Mol. Biol. Evol. 2:66-85.

Pääbo, S., Irwin, D.M. and Wilson, A.C. 1990. DNA damage promotes jumping between templates during enzymatic ampification. J. Biol. Chem. 265:4718-4721.

Pääbo, S. and Wilson, A.C. 1991. Miocene DNA sequences- A dream come true? Current Biology 1:45-46.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4:406-425.

Sidow, A., Wilson, A.C. and Pääbo, S. 1991. Bacterial DNA in Clarkia fossils. Phil. Trans. R. Soc. Lond. B 333:429-433.

Sokal, R.R. and Rohlf, F.J. 1981. "Biometry", W. H. Freeman and Company, New York.

Soltis, P.S., Soltis, D.E. and Smiley, C.J. 1992. An *rbc*L sequence from a Miocene *Taxodium* (bald cypress). Proc. Natl. Acad. Sci. USA 89:449-451.

Wilson, M.A., Gaut, B. and Clegg, M.T. 1990. Chloroplast DNA evolves slowly in the palm family (Arecaceae). Mol. Biol. Evol. 7:303-314.

Zurawski, G. and Clegg, M.T. 1987. Evolution of higher plant chloroplast DNA-encoded genes: implications for structure-function and phylogenetic studies. Ann. Rev. Plant Physiol. 38:391-418.

Zurawski, G., Clegg, M.T. and Brown, A.H.D. 1984. The nature of nucleotide sequence divergence between barley and maize chloroplast DNA. Genetics 106: 735-749.

ASSESSING THE GENETIC VARIATION IN PRE-COLUMBIAN MAIZE AT THE MOLECULAR LEVEL

F. Rollo, W. Asci and S. Sassaroli
Dipartimento di Biologia Molecolare, Cellulare e Animale,
Università di Camerino, via F. Camerini 2,
I-62032 Camerino, Italy

SUMMARY

DNA preparations obtained from two 1000-year-old maize kernels were enzymatically amplified using a polymerase chain reaction (PCR) system aimed at a 90-bp-long tract located within the conserved termini of *Mu* transposable elements and the amplification products cloned into a plasmid vector. Subsequently, the nucleotide sequences of the inserts of 33 clones were determined. Estimates of mean pairwise difference due to base substitutions inside each group of *Mu* terminal sequences, gave the following figures : 6.9 +/- 3.7 and 7.6 +/- 4.0 (referring to a 40 bp long tract) with a maximum observed difference of 14 substitutions. These estimates are very close to those obtained from the analysis of 33 *Mu* terminal nucleotide sequences from a present-day race of Peruvian maize and a *Mutator* line (resp. 7.3 +/- 3.4 and 7.8 +/- 3.4; maximum observed difference, 14 substitutions). A parallel sequence analysis performed on 20 clones of enzymatically amplified mitochondrial DNA from the 1000-year-old maize sample, showed that this DNA, though of very low molecular weight, does not contain a proportion of site-specific modifications significantly larger than that found in modern DNA. On the other hand, the same analysis showed that, referring to the considered 40-bp-long tract, ancient maize mitochondrial DNA differs from modern DNA by a base substitution (a GC-CG transversion)

INTRODUCTION

Maize Origin and Domestication

Basically, two theories have been formulated on the origin of maize: the so-called "teosinte theory" (Beadle, 1939) and the "wild maize" (Mangelsdorf, 1974) theory. According to the former, teosinte, a native grass growing in Mexico and Guatemala, and in particular the Mexican annual teosinte races, *Zea mays* subsp. *mexicana* (Schrader) Kuntze, and *Zea mays* subsp. *parviglumis* Iltis & Doebley should be considered the direct ancestors of maize. In contrast, the "wild maize" theory emphasizes the enormous difference in morphology between the ears of maize and teosinte: the former cannot have been linearly derived from the latter in so short a time as the archaeological evidence seems to suggest. The "wild maize" theory implies the existence in the past of a "proto- or wild maize" genetically distinct from teosinte. Once domesticated and crossed with a perennial teosinte (*Zea diploperennis diploperennis* Iltis Doebley & Guzman, *Zea perennis perennis* Hitchcock), the "proto maize" would have given rise to the annual teosinte races. A weak point of this theory is that no maize plant displaying genuine "wild" traits has ever been found.

A corollary of the "teosinte theory" is that maize was domesticated within or near the geographic range of teosinte, Mexico and Guatemala (Doebley, 1990). The "wild maize" theory, on the other hand, leaves some room to the possibility that maize

originated outside Meso- and Central America. In particular, the Andean regions have been repeatedly proposed as a possible "homeland" for maize by the followers of the Mangelsdorf's school (Bonavia and Grobman, 1989). Finally, whether maize has undergone multiple independent domestications or is the product of a unique evolutionary event and whether all of the morphological and physiological variations now observed among modern races of maize can be explained within the limit of normal mutation rates or whether some unknown mechanism of accelerated evolution should be invoked are all important issues which are currently matter of debate among scholars.

Origin of the *Mu* Family

Transposable elements have been shown to play an important role in plant evolution as a source of sequence divergence (Schwarz-Sommer et al., 1985). Indian corn or maize (*Zea mays* ssp. *mays* L.) is known to host several families of transposable elements, among which are the so-called *Mu* elements (Robertson, 1978). All known *Mu* elements are characterized by similar terminal inverted repeats of about 200 bp in length. Typically, one maize haploid genome contains about 40-70 copies of these terminal repeats (Chandler et al., 1986). While the first archaeological evidence about maize domestication in the Tehuacán valley (Mexico), is approximately 7000-year-old (Mangelsdorf, 1974), estimates of the age of the *Mu* transposable elements, carried out on the basis of the sequence divergence of the *Mu* termini in modern maize DNA, suggest that these elements could be $\geq$ 29 million year-old (Talbert and Chandler, 1988; Talbert et al., 1989). The recent achievements of diachronic genetics, have shown the possibility of verifying, at least to some extent, the evolutionary hypothesis formulated through the molecular analysis of the genome of present-days organisms, by the analysis of the DNA isolated from ancient remains (Pääbo, 1989). Previous studies performed in our laboratory (Rollo, 1985; Rollo et al., 1991; Venanzi and Rollo, 1990) demonstrated that plant seeds from archaeological excavations can contain low-molecular-weight ribosomal RNA (rRNA) and DNA, both of endogenous origin, and that specific DNA tracts can be retrieved and characterized using PCR (Saiki et al., 1988). In particular, the analysis of the DNA isolated from a maize sample found in a Peruvian archaeological site (Huari culture) and radiocarbon dated 980 +/- 95 years (reference year: 1950) showed the possibility of recovering short (40-bp-long) DNA sequences strongly resembling modern *Mu* termini (Rollo et al., 1991). In the present investigation we have performed an exhaustive survey of this type of sequence in ancient and modern maize samples. The study has been completed by the evaluation of the site-specific modifications affecting ancient maize DNA.

MATERIALS AND METHODS

Plant Material

Pre-Columbian maize was provided by Centro Studi Ricerche Ligabue (Venice). The plant remains come from a Huari burial site (Peruvian Coast) and are composed of nine well-preserved maize ears kept in a feather decorated canvas bag. Radiocarbon-age determination indicated an age of 980 +/- 95°C-14 years (reference year: 1950) for the maize sample.

Kernels of a modern Peruvian maize race ("Morado") were a gift of Luigi Piacenza, Associazione Italiana Studi Americanistici, Genua.

Maize kernels from a *Mutator* (Robertson, 1978) stock were provided by Norberto Pogna, Istituto Sperimentale per la Cerealicoltura, S. Angelo Lodigiano, Italy.

Nucleic Acids Extraction and Purification

Nucleic acids were phenol extracted from individual kernels of ancient or modern maize as previously reported (Rollo et al., 1991). In the case of archaeological specimens, to eliminate polymerase inhibitors, ethanol-precipitated nucleic acids were further purified by electrophoresis on a 10 x 7 x 0.3 cm 2.5% low-melt agarose minigel containing 0.5 µg/ml ethidium bromide in 1 x TBE buffer (1 x TBE: 90 mM boric acid, 2.5 mM Na_2EDTA pH 8.0). The gel was run at 80 V for 1 h. At the end of the run, the gel was put on a UV-light transilluminator and the whole fluorescent nucleic acid band excised and stored in a freezer (-25°C).

DNA Enzymatic Amplification

For the enzymatic amplification of ancient DNA, the agarose fragment containing the fluorescent nucleic acid band was melted and 1 µl of the agarose nucleic acid suspension was directly added to the PCR mixture. In control experiments, 1 µg of calf thymus DNA was gel fractionated and the agarose DNA suspension added to the PCR mixture as above described. For the analysis of modern maize samples, 1 *µl* of maize DNA, resuspended in sterile distilled water at an approximate concentration of 1 µg/ml, was directly added to the PCR mixture.

The reaction mixture (50 µl) contained 16.6 mM-$(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8 (at 25°C), 6.7 mM-$MgCl_2$, 10 mM-b-mercaptoethanol, 200 µM each of dATP, dCTP, dGTP, dTTP, 0.05% (v/v) detergent W-1 (Bethesda Research Laboratories, Gaithersburg, MD), 0.3 µg each of amplification primers, about 25 ng of gel-purified nucleic acid and 2.5 units Taq polymerase (Bethesda Research Laboratories, Gaithersburg, MD). The amplification was performed in 500 µl Eppendorf type vials using a Perkin Elmer-Cetus "Thermal Cycler" (Perkin Elmer, Norwalk, Conn., USA). The amplification conditions were set at 95°C for 1 min (DNA denaturation), 55°C for 20 sec (oligonucleotide annealing) and 72°C for 15 sec (elongation). The number of cycles ranged from 20 to 50 according to the different experiments. The following oligonucleotides were employed:

Mu termini:

TGTTTACGCAGCCCCAAGTGCTGTC, GGCGTTGGCGTTGGCTTCTCTGTTT

Cytochrome c oxydase subunit I gene:

ACTTTTGCACCGAAGAAACTCATAA, GGCTAATTCCGATACGAATACCAAA

Molecular Cloning and Sequence Analysis of Amplified DNA

PCR amplified DNA was fractionated on 2.5% low-melt agarose containing 0.5 µg/ml ethidium bromide and the relevant (90bp) amplification band visualized on a UV transilluminator and excised. The agarose fragment was melted at 60°C and the DNA phenol extracted , ethanol precipitated, desiccated and resuspended in sterile distilled water. Approximately 100-300 ng of amplified DNA were phosphorylated using 3 units of polynucleotide kinase, repaired using 2 units T_4 DNA polymerase and eventually cloned into plasmid pUC13 which had been previously cut into the *SmaI* site of the polylinker and de-phosphorylated. Individual clones were sequenced using M13 primers and the dideoxy chain termination method adapted to supercoiled plasmid DNA sequencing (Chen and Seeburg, 1985).

Precautions to Minimize Contaminations

To minimize contaminations due to DNA carryover in amplification experiments, the following precautions were taken: (i) positive-displacement pipetting devices were employed and reagents stored separately in small aliquots; (ii) gel electrophoresis equipment (gel tank and tray) used to fractionate nucleic acids isolated from the ancient specimens had not been previously used for the separation of modern plant DNAs; (iii) proper control extractions and amplification reactions were performed, using DNA preparations not expected to carry the target sequences, i.e., calf thymus DNA or DNA purified from soil samples; (iv) no purification and/or enzymatic amplification of modern plant DNA was carried on in the laboratory where ancient samples were manipulated; (v) purifications and amplifications of DNA from modern maize samples were started only after all the amplification and sequencing experiments involving ancient DNA had been carried out.

Analysis of Sequence Results

Nucleotide sequences were aligned using the MicroGenie (Beckman, Palo Alto, Ca) program. Pairwise comparisons were carried out manually and triangular matrices derived. The results were further elaborated using Systat (Systat, Evanston, IL) software.

RESULTS

Availability of template DNA

Previous investigations (Rollo et al., 1991) have shown that nucleic acids isolated from 1000-year-old Huari kernels are composed of short ($\leq$140 base pairs in length) rRNA (>90%) and DNA fragments. By the use of the 90 bp long PCR system described in Materials and Methods, DNA sequences very close to the terminal repeats common to all known *Mu* transposable elements can be obtained. To verify whether the number of ancient DNA molecules which can act as a template for the PCR amplification of the *Mu* termini is large or small, nucleic acids isolated from a single kernel were submitted to 25, 30 and 40 cycles of amplification. The results (Figure 1) show that the expected 90-bp-long amplification band is obtained by applying as little as 25 cycles of amplification. This demonstrates that the number of ancient DNA molecules which can act as a substrate for the DNA polymerase is rather large.

Analysis of *Mu* Termini in Ancient and Modern Maize

Nucleic acids obtained from two kernels from different ears of Huari maize were submitted to 40 cycles of enzymatic amplification using the 90-bp-long PCR system aimed at the right terminal repeat of *Mu1* (*Mu1R*: Talbert et al., 1989). The products of the amplification reactions were separately cloned into pUC13 and the resulting two DNA libraries screened for the presence of recombinant clones carrying inserts of a suitable size ($\geq$ 90 bp). Selected clones were further analyzed by nucleotide sequencing using M13 primers. The same procedure was repeated using, as substrates for the amplification reaction, the DNAs from a modern race of Peruvian maize ("Morado") and a *Mutator* stock (Robertson, 1978). Nucleotide sequences (40bp) were aligned with the corresponding tract of *Mu1R*, subsequently for each group of sequences, mean pairwise

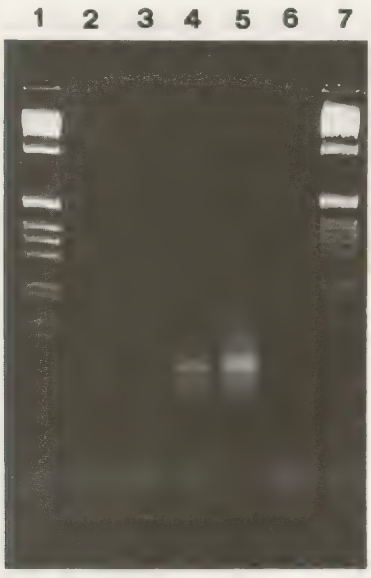


Figure 1. Enzymatic amplification of the DNA isolated from 1000-year-old maize using the 90-bp-long *Mu* terminal PCR system described in the text. Amplification products were fractionated on a 2.5% agarose minigel and the gel treated with ethidium bromide and photographed under UV light. 1,7 DNA size marker ("1 kb ladder" Bethesda Research Laboratories); 2-5, 1000-year old maize DNA (20, 25, 30 and 40 amplification cycles respectively); 6, calf thymus DNA (40 amplification cycles).

differences due to base substitutions were calculated. The results are summarized in Table 1 .

Table 1. Estimates of pairwise divergence due to base substitutions for 40-bp-long *Mu* terminal sequences from ancient (1000-year-old) and modern maize kernels

Sample age	Stock / source	Sample no.	Mean pairwise divergence (base substitutions ±SE)	Max. divergence (base substitutions)
Ancient	Huari	4	6.9 + 3.7	14
	Huari	6	7.6 + 4.0	14
Modern	"Morado"	-	7.3 + 3.4	14
	Mutator	-	7.8 + 3.4	14

Finally, pairwise differences between *Mu* termini in Huari (kernel no 4) and modern ("Morado") maize were determined and the results further elaborated to produce a dendrogram, using nearest neighbor analysis (Figure 2).

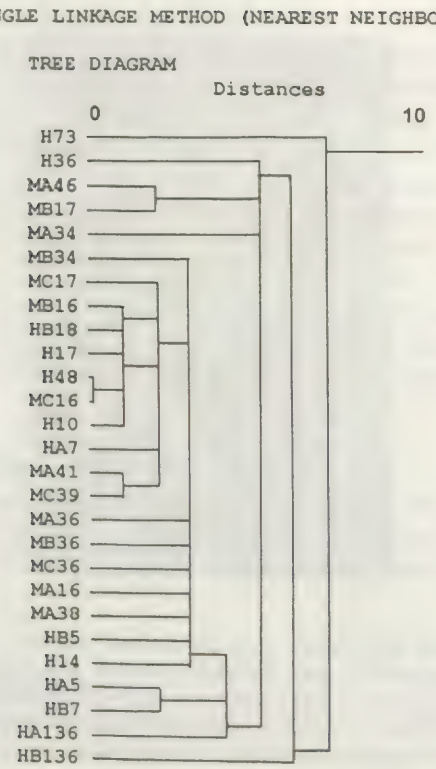


Figure 2. Nearest neighbor analysis of the pairwise differences due to base substitutions between Huari (kernel no. 4) and modern ("Morado") *Mu* termini.

Site-Specific Modifications Affecting the Ancient Double Helix

It is known that products of the PCR-mediated amplifications of DNA contain errors (mutations) mostly in the form of base-substitutions. Errors are caused by two distinct phenomena: i) intrinsic properties of the polymerase employed for the amplification; ii) state of the template DNA (Eckert and Kunkel, 1991). When freshly prepared DNA from modern specimens is used, errors are almost exclusively due to the intrinsic properties of the polymerase (Taq DNA polymerase, Vent ®, T7 DNA Polymerase, etc.) utilized. On the other hand, when the template for the amplification reaction is ancient DNA, the effect of the site-specific damage, such as modified bases, apurinic or apyrimidinic (Ap sites) and strand fragmentation, adds significantly (Pääbo et al., 1990). Hence, by comparing the number of errors generated by the amplification of modern and ancient DNA preparations, one can, in principle, obtain indications about the consistence of the damage affecting the latter. The effect of site-specific damage on DNA sequences retrieved from ancient specimens should be taken into account in order to make precise comparisons between ancient and present-day DNA sequences. With this in view, as a control experiment for the incidence of errors affecting the amplification products of modern DNA, a recombinant plasmid carrying a 90 bp long DNA tract corresponding to a *Mu* terminal sequence (plasmid pCho33) was submitted to 50 amplification cycles. Amplified DNA was then cloned, and the nucleotide sequence

of 27 individual clones determined. This analysis only showed a single base substitution (an AT-GC transition) affecting one clone (estimated error rate per nucleotide per cycle: p = 1/27,000, calculated using the formula $p = 2f/n$ where f is the observed error frequency per nucleotide and n the number of cycles, Eckert and Kunkel, 1991). To determine the error frequency for 1000-year-old DNA, a PCR system spanning from nucleotide -180 to nucleotide -91 of the maize mitochondrial cytochrome c oxydase subunit I gene (Isaac et al., 1985) was employed. Nucleic acids isolated from a kernel of Huari maize were submitted to 40 + 20 cycles of enzymatic amplification using the mitochondrial PCR system, and the amplification products cloned and sequenced. The comparison of the nucleotide sequences of 20 individual clones (800 nucleotides in total) showed a single GC-AT transition (estimated error rate per nucleotide per cycle: p = 1/24,000) affecting one of the clones. On the other hand, when the 20 ancient mitochondrial sequences were aligned with the published (Isaac et al., 1985) sequence of the modern gene, all evidenced an identical base substitution, a GC-CG transversion corresponding to the nucleotide -120 of the modern gene sequence.

DISCUSSION

Gene technologies can provide an insight into past genetic diversity and, implicitly, an estimate of the loss of genetic diversity through the ages. The issue is now whether significant genetic information can be retrieved only from samples containing well preserved ancient DNA, or whether this operation can be accomplished also when only poorly preserved DNA is available. Such an issue is of relevant and practical importance as most of the ancient plant samples seem to contain degraded DNA only, while samples containing well preserved DNA are extremely rare. Previous investigations performed on the 1000-year-old maize sample described in this paper (Rollo et al., 1991) have shown that kernels contain de-polymerized DNA. The use of a PCR system as short as 90bp and the choice of a mid-repetitive sequence such as the *Mu* termini (about 40-70 copies per haploid genome, Chandler et al., 1986) as a target for the amplification reaction, however, compensate the drawback posed by the poor state of preservation of the DNA. This is made evident by the fact that the application of a relatively small number (25) of amplification cycles gives rise to a distinct band on the gel. The availability of a relatively large number of template molecules makes the results of the amplification experiments reproducible and representative of the range of variation of *Mu* termini in the different samples. The analysis of HLA gene sequences performed by Lawlor et al. (1991) on soft human tissue isolated from an approximately 7500-year-old remain showed, in three of four amplifications carried out starting from the same DNA preparation, a predominant sequence variant which differed among the amplification products. Such a result is indicative that in those experiments only a very small number of DNA sequences was available as a substrate for the PCR. In our case, if we compare the *Mu*-termini variants obtained from two independent amplifications of ancient maize DNA, we can observe that no particular sequence variant is preferentially amplified, but, rather, a broad spectrum of variants is constantly obtained. This result is also confirmed by the calculation of the mean pairwise differences due to base substitutions in all the samples considered, giving fairly comparable figures, and by a previously performed characterization of the amplification products of the *Mu* terminal PCR system using single-strand conformation polymorphism (SSCP) analysis (Amici and Rollo, 1992). In the latter case, the products of independent amplification

experiments performed on the same ancient DNA preparation gave identical SSCP banding patterns. Sequence analysis of individual clones of PCR-amplified mitochondrial DNA from Huari maize reveals that substitutions due to both Taq polymerase errors and DNA damage, cannot add significantly to the figures of sequence divergence calculated for the ancient *Mu* termini. The comparison of the error rate per nucleotide per cycle in modern and 1000-year-old maize DNA indicates that the latter, though of very low molecular weight, does not carry modified sites in a larger proportion than the former. Pääbo et al. (1990) found that the DNA isolated from a 4000-year-old human remain from Egypt was significantly affected by site-specific damage (almost 4% modified sites). If we assume that damage accumulates at a constant rate, 1000-year-old human DNA should carry nearly 1% of modified sites. Clearly, this is not the case of the 1000-year-old maize DNA. At the present state of knowledge about long-time DNA preservation, no clear-cut explanation is available for this phenomenon, which seems to show a differential sensitivity of human mummy and plant seed DNAs to site-specific damage. Further evidence is required to extend this observation and verify whether it represents a general occurrence or, rather, an exception. Incidentally, the analysis of the clones of enzymatically amplified mitochondrial DNA from ancient maize has also shown that, compared to the published modern sequence (Isaac et al., 1985) of the cytochrome *c* oxidase subunit I gene, the former constantly show a base substitution (a GC-CG transversion) corresponding to the nucleotide -120 of the published gene sequence. In this case too, further work is required to determine the frequency of this haplotype in pre-Columbian maize populations and its possible presence in modern maize populations. As regards the *Mu* termini, the estimates of mean pairwise difference due to base substitutions among Huari termini are quite close to the estimates derived from modern ones. This conclusion is further stressed by the dendrogram obtained by nearest-neighbor analysis of the pairwise differences between Huari and modern termini, evidencing no separate branching of the two groups. Rather, a Huari terminal sequence can be more similar to a modern one than to another Huari terminal sequence and vice-versa.

By comparing the complete nucleotide sequences of the conserved terminal repeats of the *Mu1*, *Mu4* and *Mu5* elements, Talbert et al., (1989) calculated that *Mu* termini diverged by up to 17% of their sequence. By assuming that all *Mu* termini derived from a single ancestor and by considering a possible rate of divergence of 10^{-9} substitutions/site/year the same authors estimated that *Mu* termini in maize had to be at least 29 million years old. The present estimates indicate that sequence divergence among modern *Mu* termini can be as much as 35%. On the other hand, the maximum sequence divergence among Huari *Mu* termini (35%) indicates that in the last millennium no appreciable diversification of the *Mu* termini has taken place. This concept can be expressed in another way i.e., that the diversification of the *Mu* termini proceeds at a pace which is undetectable over a time span of 1000 years. Our diachronic investigation thus confirms and extend Talbert's results, by showing that the *Mu* termini are an extremely old component of the maize genome, certainly much older than any presently available archaeological evidence on maize origin.

ACKNOWLEDGEMENTS

The authors wish to thank Centro Ricerche Studi Ligabue for providing ancient maize samples. This research was supported by MURST.

REFERENCES

Amici, A. and Rollo, F. 1992. PCR-SSCP characterization of *Mu* element components in a Huari (10th century) maize sample. Ancient DNA Newsl. 1: 22-24.

Beadle, G.W. 1939. Teosinte and the origin of maize. J. Heredity 30:245-247.

Bonavia, D. and Grobman, A. 1989. Andean maize: its origin and domestication, *in*: "Foraging and Farming: The Evolution of Plant Exploitation," D.R. Harris and G.C. Hillman, eds., Unwin-Hyman, London.

Chandler, V., Rivin, C. and Walbot, V. 1986. Stable non-mutator stocks of maize have sequences homologous to the *Mu*1 transposable element. Genetics 114:1007-1021.

Chen, E.J. and Seeburg, P.H. 1985. Supercoil sequencing: a fast simple method for sequencing plasmid DNA. DNA 4:165-170.

Doebley, J. 1990. Molecular evidence and the evolution of maize. Econ. Bot. 44:6-27.

Eckert, K.A. and Kunkel, T.A. 1991. DNA polymerase fidelity and the polymerase chain reaction. PCR Methods Applic. 1:17-24.

Isaac, P.G., Jones, V.P. and Leaver, C.J. 1985. The maize cytochrome c oxidase subunit I gene: sequence, expression and rearrangement in cytoplasmic male sterile plants. EMBO J. 4:1617-1623.

Lawlor, D.A., Dickel, C.D., Hauswirth, W.W. and Parham, P. 1991. Ancient HLA genes from 7,500-year-old archaeological remains. Nature 349:785-788.

Mangelsdorf, P.C. 1974. "Corn. Its Origin, Evolution and Improvement", Harvard University Press, Cambridge, Massachussetts.

Pääbo, S. 1989. Ancient DNA and the polymerase chain reaction. J. Biol. Chem. 264: 9709-9712.

Pääbo, S., Irwin, D.M. and Wilson, A.C. 1990. DNA damage promotes jumping between templates during enzymatic amplification. J. Biol. Chem. 265: 4718-4721.

Robertson, D.S. 1978. Characterization of a mutator system in maize. Mutat. Res. 51:21-28.

Rollo, F. 1985. Characterization by molecular hybridization of RNA fragments isolated from ancient (1400 BC) seeds. Theor. Appl. Genet. 41:330-333.

Rollo, F., Venanzi, F.M. and Amici, A. 1991. Nucleic acids in mummified plant seed: biochemistry and molecular genetics of pre-Columbian maize. Genet. Res. Camb. 58:193-201.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-489.

Schwarz-Sommer, Z., Gierl, A., Cuypers, H., Peterson, P.A. and Saedler, H. 1985. Plant transposable elements generate the DNA sequence diversity needed in evolution. EMBO J. 3:591-597.

Talbert, L.E. and Chandler, V.L. 1988. Characterization of a highly conserved sequence related to Mutator elements in maize. Mol. Biol. Evol. 5:519-529.

Talbert, L.E, Patterson, G.I. and Chandler, V.L. 1989. *Mu* transposable elements are structurally diverse and distributed throughout the genus *Zea*. J. Mol. Evol. 29:28-39.

Venanzi, F.M. and Rollo, F. 1990. Mummy RNA lasts longer. Nature 343:25-26.

DNA IN WHEAT SEEDS FROM EUROPEAN ARCHAEOLOGICAL SITES

Terence A. Brown, Robin G. Allaby, Keri A. Brown
Kerry O'Donoghue and Robert Sallares
Department of Biochemistry and Applied Molecular Biology
UMIST, Manchester, M60 1QD, UK

SUMMARY

We have detected small amounts of ancient DNA in wheat seeds collected from four archaeological sites in Europe and the Middle East. We describe techniques that have enabled us to obtain nucleotide sequences from the preserved DNA and discuss ways in which these sequences could be used in archaeobotanical studies. We also present speculations concerning the factors that influence the decay of nucleic acids in ancient biological material.

WHEAT IN PREHISTORY

The main impetus to our work derives from the importance of wheat as a central component of prehistoric agriculture in the Old World (see Brown et al., 1993). Wheat was one of the first plants to be cultivated by the early farmers of the 'Fertile Crescent', an area of the Near and Middle East comprising the plains of Mesopotamia, the deserts of Syria, and some of the neighboring mountainous areas of Anatolia. The earliest indication of wheat cultivation comes from Tell Mureybit, on the east bank of the Euphrates in North Syria, where wild einkorn, *Triticum boeoticum* (classification of Miller, 1987), was utilized in the eighth millennium BC and possibly earlier (Bell, 1987). During the following 2000 years the number of cultivated species proliferated and domesticated forms (i.e., those not known in the wild) appeared for the first time. The genetic transformations that took place during this period led to morphological changes such as less fragile ears (an aid to harvesting as the mature seeds remain attached to the plant rather than being dispersed) and a reduction in the size of the glumes enclosing the seeds (resulting in easier threshing). Domestication was also associated with an increase in ploidy level, the first hexaploid wheat arising by amphiploidy, probably between weeds of the diploid *Aegilops squarrosa* growing in a field of tetraploid *Triticum dicoccum* somewhere in Syria in the latter part of the eighth millennium BC (Bell, 1987; Miller, 1987).

This period of genetic diversification was accompanied by an expansion in the geographical range of wheat. Farming reached the Balkans soon after 6000 BC and subsequently spread throughout Europe via the Danube and Rhine valleys, and along the Mediterranean coast, arriving in northern Europe and Britain around 4000-3500 BC (Renfrew, 1973). During roughly the same period similar though less well-studied trajectories are thought to have resulted in the expansion of wheat cultivation into central and southern Asia and the Nile valley (Harris and Hillman, 1992). The spread of farming is, in essence, a record of prehistoric population interactions, as transfer of the technology from one place to another could only have occurred through human contact, either by the farmers supplanting an indigenous population, or by a peaceful acculturation of the existing society. The spread of more 'advanced' varieties of wheat in the millennia following the initial expansion of agriculture enables these human contacts to be studied through an extensive period of prehistory, not just during the centuries

when farming first became established.

Changing relationships among human populations could therefore be studied by examining the types of wheat grown at different archaeological sites. In trivial terms, the presence of closely related wheat at two or more contemporaneous sites would imply contact, whereas widely differing wheat would suggest cultural distinctiveness. Unfortunately it is difficult to address these possibilities by morphological studies alone. Only with the 'glume wheat' (those with which the chaff tightly encloses the grain) can species and varieties be discriminated with any confidence from the morphological features of the parts of the plants normally preserved on archaeological sites. The 'naked wheat', which include agriculturally important varieties such as *Triticum turgidum, Triticum durum* and *Triticum aestivum*, are often impossible to distinguish. With these wheat the morphological indicators are so non-diagnostic that it may not even be possible to fix the ploidy level of an archaeological specimen. This imprecision in genotype identification limits the confidence that can currently be assigned to archaeobotanical based analyses of human population interactions.

ANCIENT DNA IN ARCHAEOLOGICAL WHEAT

To exploit the potential of wheat as a means of studying interactions between prehistoric communities we need more reliable methods for examining the genotypes of archaeological remains. The greatest accuracy in genotype analysis is provided by comparisons of nucleotide sequence polymorphisms. DNA analysis has revolutionized the study of extant populations of wheat and other plants, and could do the same for archaeological material, if DNA is preserved in a quantity and form suitable for sequence analysis. The first indications that ancient DNA might be present in archaeological plant remains came from studies with preserved maize ears (Goloubinoff et al., 1993; Helentjaris, 1988; Rollo et al., 1988; Rollo et al., 1991). The successful extraction of ancient DNA from this type of material prompted us to attempt similar experiments with wheat.

Wheat Used in This Study

The four principal means by which plant remains survive the processes of decay are: charring, involving partial or complete reduction to carbon by heat; the exclusion of water in desiccating environments; the exclusion of oxygen in anoxic, often waterlogged environments; and partial mineralization by calcium phosphate, calcium carbonate or, less commonly with archaeological remains, iron sulfate (pyrites). We have assessed the survival of DNA in wheat representative of each of the preservation modes (Table 1). Charring is the most important form of preservation in archaeological terms because of the widespread occurrence of charred plant remains in varied archaeological contexts. Much of our work to date has been with charred *T. spelta* from the Iron Age hillfort at Danebury, Hampshire, UK (Jones, 1984; Nye and Jones, 1991), because material available from this site is plentiful and protected against aerial contamination during collection. *T. spelta* is a hexaploid wheat; as a comparative tetraploid we have used charred *T. dicoccum* grains from the equally rich and well sampled site at Assiros Toumbos in northern Greece (Jones and Halstead, 1980). Waterlogged cereals are less commonly preserved but we have been able to study a mediaeval sample of *T. aestivum* from Oxford City, UK, and are collaborating with S. Jacomet of Basle who is working extensively on wet-preserved wheat from the famous Swiss Lake Village sites. Finally,

Table 1. Wheat remains used in this study. BP = before present.

Species	Preservation	Age (BP)	Site
T. spelta	charred	2000 years	Danebury, UK
T. dicoccum	charred	3300 years	Assiros, Greece
T. aestivum	waterlogged	700 years	Oxford, UK
T. dicoccum	desiccated/ mineralized	1000 years	Tell Es Sa'idiyeh, Jordan

mediaeval contexts from the site of Tell Es Sa'idiyeh in Jordan have provided partially desiccated, partially phosphate mineralized grains of *T. dicoccum.*

Methodology

Plant nucleic acids were extracted by a modification of a standard procedure (Rogers and Bendich, 1985). After grinding, 500 mg of seeds were suspended in 1 ml of extraction buffer (2% cetyl trimethylammonium bromide [CTAB], 100 mM Tris-HCl pH 8.0, 20 mM disodium EDTA, 1.4 M NaCl) and incubated at 60°C for 30 min. Debris was removed by centrifuging in a microfuge for 3 min, and the supernatant extracted twice with 24:1 chloroform : isoamyl alcohol. The aqueous layer, containing nucleic acids, was added to 2 vols precipitation buffer (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM disodium EDTA) and incubated at 4°C for 1 h. The precipitated nucleic acids were pelleted by centrifugation for 8 min and resuspended in 50 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM disodium EDTA). The sample was then electrophoresed in a 1.3% agarose gel and material corresponding to double stranded DNA in the size range 100 to 1000 bp excised, placed in dialysis tubing with 450 ml 0.2X TBE buffer (17.8 mM Tris-borate pH 8.3, 0.4 mM disodium EDTA) and nucleic acids electroeluted at 7 V cm^{-1} for 1 h, followed by reversed current for 2 min. The nucleic acid solution was removed from the dialysis tubing and stored at -20°C.

Standard methods were used for hybridization analysis (Ausubel et al., 1993). Electroeluates were immobilized on a nylon membrane (BioRad ZetaProbe) by dot-blotting and the membrane prehybridized for 5 h at 65°C in 50 ml of 5X SSPE (0.9 M NaCl, 0.05 M NaH_2PO_4, 0.005 M disodium EDTA), 5X Denhardts (0.1% bovine serum albumin; 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), 1% sodium dodecyl sulfate, 100 mg ml^{-1} denatured salmon sperm DNA. The membrane was hybridized overnight at 65° C with a radiolabelled restriction fragment of wheat chloroplast DNA in 50 ml 6X SET (0.9 M NaCl, 0.12 M Tris-HCl pH 7.8, 6 mM disodium EDTA), 10X Denhardts, 0.1% sodium dodecyl sulfate. After hybridization the membrane was washed four times for 10 min each in 50 ml 6X SSPE, 0.1% sodium dodecyl sulfate at the hybridization temperature and bound probe detected by autoradiography.

Polymerase chain reactions (PCRs) were carried out in 100 ml reaction mixes containing 10 ml electroeluate, 10 ml 10X buffer (Boehringer-Mannheim), 50 mM each

of dATP, dTTP, dGTP and dCTP, 2 mM $MgCl_2$, 200 ng of each 20-mer primer, and 2.5 units *Taq* DNA polymerase (Boehringer-Mannheim). Cycle times for the glutenin primers (see Figure 1) were: 2.5 min at 94°C; 30 cycles of 2 min at 60°C, 1 min at 74°C, 1 min at 94°C; 2 min at 60°C; 9 min at 74°C. Twenty five ml of each reaction was fractionated in a 3% NuSieve (FMC BioProducts) agarose gel and the PCR products visualized by ethidium bromide staining and U.V. transillumination.

Results of the Ancient DNA Studies

Hybridization analyses were carried out with extracts of the Danebury, Oxford and Tell Es Sa'idiyeh seeds and signals indicating to the presence of wheat nucleic acids obtained in each case. These experiments did not provide precise quantifications of the amounts of nucleic acid present in the extracts, but the sensitivity of the technique is such that >50 ng nucleic acid per g seed would be needed to see a hybridization signal with the procedure that was used. Whether the signals derive from the presence of DNA, RNA, or a combination of both, has not been determined. Our procedure should have resulted in the preferential immobilization of DNA, but the presence of substantial amounts of RNA in preserved maize seeds (Rollo et al., 1991) means that we cannot discount the possibility that our signals are due to RNA.

PCRs targeted at a 246 bp region immediately upstream of the genes for the glutenin proteins were carried out to gain further information on the nucleic acids extracted from the Danebury and Assiros Toumbos seeds. The results of one such PCR are shown in Figure 1; although these experiments have been inconsistent, some extracts

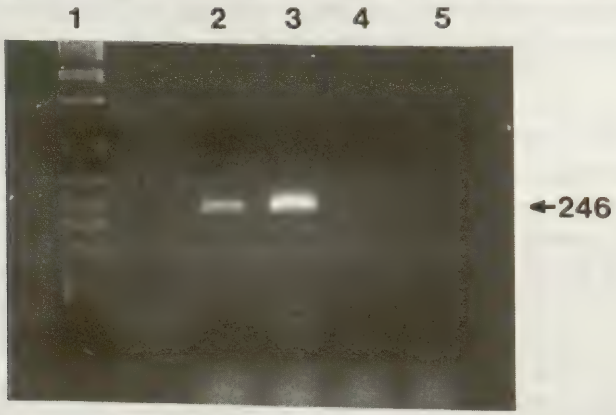


Figure 1. PCR analysis of an extract from the Danebury wheat seeds. Lanes: 1, DNA size markers; 2, result of PCR with modern *T. aestivum* DNA; 3, result of PCR with an extract from the charred *T. spelta* seeds; 4, result of PCR with an extract blank (extraction procedure carried out with no seeds); 5, result of PCR with a water blank (PCR set up with no DNA). The position of the 246 bp band is indicated.

failing to produce a PCR product and others giving multiple products of unexpected sizes, the results suggest that at least some of the seeds in the preserved samples contain DNA..

Exploitation of Ancient DNA in Archaeological Wheat

Our experiences with ancient DNA from bones (Brown et al., 1993) have shown

us that the initial detection of DNA is relatively easy, but that subsequent exploitation of the genetic resource in studies of archaeological relevance can be much more difficult. Our aim in the wheat project is to build up a genetically based phylogeny of archaeological wheat and to use this phylogeny to deduce the relationships among plants grown at different geographical locations and at different times. This information will point towards the original domestication event(s) and allow the initial trajectories for the spread of wheat cultivation, as well as the later interactions between farming communities, to be traced. Our most important consideration when embarking on this work is to chose a genomic region that displays a level of polymorphism appropriate to the questions that we are asking. With human material we could use mitochondrial DNA sequences (e.g., Cann et al., 1987), *HLA* polymorphisms (e.g., Lawlor et al., 1991) and/or CA repeats (e.g., Hagelberg et al., 1991b) to separate populations and infer the relationships between them.

Although biotechnological considerations have prompted a recent surge of research activity, especially with crops such as wheat, genomic polymorphisms are in general much less well documented in plants compared with animals. Our choice is further limited by the probable inapplicability of Random Amplified Polymorphic DNA (RAPD) analysis, an established PCR-based method for studying genetic polymorphisms that has been applied to a number of organisms including *Triticum* species (Vierling and Nguyen, 1992; Weining and Langridge, 1991). RAPD primers have random sequences and amplification results when primers anneal to opposite strands at relatively close positions (up to about 3Kb). Different amplification events result in a banding pattern (the genomic loci may not be known, and in fact are not relevant) that are reproducible for a single individual. Differences between the banding patterns for two individuals of the same species indicate nucleotide sequence polymorphisms. The technique is probably the most powerful yet devised for the type of phylogenetic analysis that we wish to carry out.

Unfortunately, as with other types of ancient material, the DNA present in the wheat seeds we have studied is broken into fragments with an average length of <1000 bp. This limits the amount of information obtainable by RAPD analysis and provides an added complication in that 'jumping PCR' (Pääbo et al., 1990) might be expected to produce additional spurious bands if amplification of such short molecules is carried out with random primers. As far as we are aware RAPD analysis has not been attempted with ancient DNA from any source so our arguments have not been tested. In fact plant seeds (especially charred ones) are probably the only type of preserved material to which the technique is theoretically applicable as most others are significantly contaminated with microbial DNA (Brown and Brown, 1992; Hagelberg et al., 1991a).

Despite these gloomy prognostications it is possible from the literature to identify polymorphic loci in the wheat genome that might be suitable for the phylogenetic studies we wish to carry out. Our work at present is aimed at assessing the potential of these loci with modern populations of wild wheat. Once we have distinguished a suitable region we will apply the relevant PCR system to the ancient remains.

FACTORS INFLUENCING THE DECAY OF DNA IN ANCIENT BIOLOGICAL MATERIAL

Ancient DNA has now been detected in a variety of types of preserved material: bones (e.g., Hagelberg et al., 1989), mummified tissues (e.g., Pääbo, 1985), skins preserved by taxidermy (e.g., Higuchi et al., 1984), insects in amber (e.g., DeSalle et al.,

1992), as well as plant remains. The only specimens that have generally failed to produce ancient DNA are bog bodies (Hughes et al., 1986; Osinga et al., 1992), this absence being expected as the highly acidic or alkaline conditions in most bogs should readily cleave phosphodiester bonds within DNA molecules. There are exceptions, however, with ancient DNA having been obtained from bodies in the bogs at Little Salt Spring (Pääbo et al., 1988) and Windover (Lawlor et al., 1991) in Florida, the explanation being that the combination of an acid bog resting on a limestone base has led to neutral conditions at the bottom of the bog. This particular case may be explainable but others are not. There are unconfirmed reports of ancient DNA in museum specimens that have been pickled in formalin for several decades: this should be impossible as formalin has a specific and rapid reaction with adenine nucleotides leading to dimers that should prevent subsequent amplification by PCR (Blackburn, 1990).

Even if the formalin report turns out to be incorrect there are still a number of corroborated observations that completely overthrow our preconceptions concerning DNA degradation in biological material. This debate has been energetically pursued by Pääbo and Wilson (1991), who had difficulty coming to terms with the existence of ancient DNA in a 16-million-year old *Magnolia* leaf from the Clarkia deposits (Golenberg, 1991; Golenberg et al., 1990). Their argument was based on extrapolations from studies of the breakdown of DNA in aqueous solutions at elevated temperatures (Lindahl and Andersson, 1972; Lindahl and Nyberg, 1972) which suggested that fragments of DNA of the size detected in the *Magnolia* leaf (800 bp) should not survive preservation for more than 5000 years. The arguments presented by Pääbo and Wilson (1991) look sound on paper, which tells us that DNA in preserved biological material does not degrade in the same way as DNA in aqueous solutions. Based on our current knowledge, is it possible to speculate as to the factors that *do* influence DNA decay in preserved biological material?

First we should ask whether decay is linear or non-linear over time. If the former then the fact that the chromosomal DNA molecules in wheat seeds are reduced to <1000 bp after 2000 years indicates that seeds only a few thousand years older will have essentially no amplifiable DNA. Fortunately all the available evidence points to a non-linear decay of DNA, with the average sizes of the preserved molecules in dried animal tissues not changing to any great extent between four years and several thousand years after death (Pääbo, 1989). Our own results show that the absolute amount of ancient DNA is not appreciably different in bones 200 and 9000 years old (Brown et al., in press). One can speculate that DNA damage might occur relatively rapidly in the period immediately following death, due to disruption of normal cellular processes leading to release into the nucleus of DNA degrading enzymes and reactive groups. These autolytic processes should, however, be halted by post-mortem events such as drying or removal of oxygen. The latter almost certainly accounts for the preservation of DNA that is relatively undamaged in leafs from the Clarkia deposits (Golenberg, 1991), and the general argument probably holds true for all 'exceptionally-preserved' biological remains such as mummies and museum skins, where preservation of DNA is associated with at least partial retention of the overall biological structure.

These arguments also help us understand why DNA is present in charred wheat seeds. These seeds became charred by burning, a result of deliberate, accidental or spontaneous combustion of the grain stores. The seeds were probably reduced quite rapidly to the state in which they are recovered. The implication is that autolytic processes within the seeds were halted abruptly: indeed it could be argued that as the

seeds were still viable when burnt essentially no autolysis would have taken place. This is not certain as complete burning (i.e., complete reduction to elemental carbon) would per se lead to complete destruction of the DNA. We suspect that the DNA is retained in pockets of unreduced cellular debris within the charred superstructure of the seed. The critical factor is probably the loss of water, driven out of the seed by the effects of burning, as this would have terminated the activities of autolytic enzymes and probably suspended other chemical reactions as well. The fact that these seeds have remained dry during preservation is clear from the fact that their morphological structure disappears as soon as they are rehydrated - in other words they fall apart if you drop them in water.

One additional point is worth making. The immobilization of nucleic acids on inorganic matrices such as apatite is well known and formed the basis to some of the early methods for DNA extraction from biological material. We know virtually nothing about the nature of the complex formed between the inorganic matrix and the DNA, and indeed do not as yet have techniques suitable for studying supramolecular structures of this nature. Is it possible that DNA in an inorganic matrix (e.g., bone, burnt plant material, mineralized fossil material) might be present in a form that actively promotes its survival?

CONCLUSION

To gain a better understanding of the factors that influence DNA preservation, we need more data: exactly what types of chemical and physical modifications occur in DNA molecules preserved under different conditions? are other biomolecules also preserved in material such as the Clarkia leaves or is the preservation of DNA in itself exceptional? can we extract ancient DNA from seeds and other 'unexceptional' plant remains older than those that have been studied so far? Even partial answers to these questions would help us understand where to look for ancient DNA and how to ensure that our artificially preserved specimens in herbaria and museums retain their genetic material.

ACKNOWLEDGEMENTS

Our work on archaeological wheat is a collaborative project with Martin Jones and Chris Howe (University of Cambridge, UK). We thank Glynis Jones (University of Sheffield, UK), Mark Robinson (University of Oxford, UK) and Alan Clapham (University of Cambridge, UK) for providing us with preserved wheat seeds. RGA and KAB are research assistants funded by the Natural Environment Research Council; KO'D is a research assistant funded by the Science and Engineering Research Council; RS holds a Royal Society postdoctoral fellowship.

REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. Eds. 1993. "Current Protocols in Molecular Biology," John Wiley, New York.

Bell, G.D.H. 1987. The history of wheat cultivation, *in:* "Wheat Breeding - Its Scientific Basis," F.G.H. Lupton, ed., Chapman and Hall, London.

Blackburn, G.M. 1990. Covalent interactions of nucleic acids with small molecules, *in:* "Nucleic Acids in Chemistry and Biology," G.M. Blackburn and M.J. Gait, eds., IRL Press, Oxford.

Brown, K.A., Allaby, R.G., Garland, A.N. and Brown, T.A. 1993. Ancient DNA in archaeological bones and plant remains, *in:* "Archaeological Sciences 1991 Proceedings," J.Szymanski, ed. (in press).

Brown, T.A., Allaby, R.G., Brown, K.A. and Jones, M.K. 1993. Biomolecular archaeology of wheat: past, present and future. World Archaeol. 25:64.

Brown, T.A. and Brown, K.A. 1992. Ancient DNA and the archaeologist. Antiquity 66:10.

Cann, R.L., Stoneking, M. and Wilson, A.C. 1987. Mitochondrial DNA and human evolution. Nature 325:31.

DeSalle, R., Gatesy, J., Wheeler, W. and Grimaldi, D. 1992. DNA sequences from a fossil termite in Oligo-Miocene amber and their phylogenetic implications. Science 257:1933.

Golenberg, E.M. 1991. Amplification and analysis of Miocene plant fossil DNA. Phil. Trans. R. Soc. Lond. B 333:419.

Golenberg, E.M, Giannasi, D.E., Clegg, M.T., Smiley, C.J., Durbin, M., Henderson, D. and Zurawski, G. 1990. Chloroplast DNA sequence from a Miocene *Magnolia* species. Nature 344:656.

Goloubinoff, P., Pääbo, S. and Wilson, A.C. 1993. Molecular characterization of ancient maize: potentials and pitfalls, *in:* "Corn and Culture in the Prehistoric New World," S. Johannesen and C.A. Hastorf, eds., Westview Press, Boulder (In press).

Hagelberg, E., Bell, L.S., Allen, T., Boyde, A., Jones, S.J. and Clegg, J.B. 1991a. Analysis of ancient bone DNA: techniques and applications. Phil. Trans. R. Soc. Lond. B 333: 399-407.

Hagelberg, E., Gray, I.C. and Jeffreys, A.J. 1991b. Identification of the skeletal remains of a murder victim by DNA analysis. Nature 352:427.

Hagelberg, E., Sykes, B. and Hedges, R. 1989. Ancient bone DNA amplified. Nature 342:485.

Harris, D.R., and Hillman, G.C. Eds. 1992. "Foraging and Farming." Unwyn, London.

Helentjaris, T. 1988. Does RFLP analysis of ancient Anasazi samples suggest that they utilize hybrid maize? Maize Genet. Coop. News Let. 62:104-105.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A. and Wilson, A.C. 1984. DNA sequence from the quagga, an extinct member of the horse family. Nature 312:282.

Hughes, M.A., Jones, D.S. and Connolly, R.C. 1986. Body in the bog but no DNA. Nature 323:208.

Jones, G.E.M. and Halstead, P. 1980. Bioarchaeological remains from Assiros Toumbos. Annu. Brit. Sch. Ath. 75:265.

Jones, M.K. 1984. The plant remains, *in*: "Danebury: an Iron Age Hillfort in Hampshire," B. Cunliffe, ed., Council for British Archaeology Research Report 52, London.

Lawlor, D.A., Dickel, C.D., Hauswirth, W.W. and Parham, P. 1991. Ancient HLA genes from 7,500-year-old archaeological remains. Nature 349:785.

Lindahl, T. and Andersson, A. 1972. Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. Biochemistry 11:3618.

Lindahl, T. and Nyberg, B. 1972. Rate of depurination of native deoxyribonucleic acid. Biochemistry 11:3610.

Miller, T.E. 1987. Systematics and evolution, *in*: "Wheat Breeding - Its Scientific Basis," F.G.H. Lupton, ed., Chapman and Hall, London.

Nye, S. and Jones, M.K. 1991. The plant remains: a quantitative analysis of crop debris, *in*: "Danebury: an Iron Age Hillfort in Hampshire," B.Cunliffe, ed., Council for British Archaeology Research Report 73, London.

Osinga, J., Buys, C.H.C.M. and van der Sanden, W.A.B. 1992. DNA and the Dutch bog bodies. Ancient DNA Newsletter 1(2):21.

Pääbo, S. 1985. Molecular cloning of Ancient Egyptian mummy DNA. Nature 314:644.

Pääbo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proc. Natl. Acad. Sci. USA 86:1939.

Pääbo, S. and Wilson, A.C. 1991. Miocene DNA sequences - a dream come true? Curr. Biol. 1:45.

Pääbo, S., Gifford, J.A. and Wilson, A.C. 1988. Mitochondrial DNA sequences from a 7000-year old brain. Nucl. Acids Res. 16:9775.

Pääbo, S., Irwin, D.M. and Wilson, A.C. 1990. DNA damage promotes jumping between templates during enzymatic amplification. J. Biol. Chem. 265:4718.

Renfrew, J.A. 1973. "Palaeoethnobotany. The Prehistoric Food Plants of the Near East and Europe." Methuen, London.

Rogers, S.O. and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. Plant Molec. Biol. 5:69.

Rollo, F., Amici, A., Salvi, R. and Garbuglia, A. 1988. Short but faithful pieces of ancient DNA. Nature 335:774.

Rollo, F., Venanzi, F.M. and Amici, A. 1991. Nucleic acids in mummified plant seeds: biochemistry and molecular genetics of pre-Columbian maize. Genet. Res. 58:193.

Vierling, R.A. and Nguyen, H.T. 1992. Use of RAPD markers to determine the genetic diversity of diploid, wheat genotypes. Theor. Appl. Genet. 84:835.

Weining, S. and Langridge, P. 1991. Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. Theor. Appl. Genet. 82:209.

PHYLOGENETIC AND TAXONOMIC INFORMATION FROM HERBARIUM AND MUMMIFIED DNA

Scott O. Rogers
Faculty of Environmental and Forest Biology
State University of New York
College of Environmental Science and Forestry
Syracuse, New York 13210, USA

SUMMARY

In Part I, results are presented from a comparison of nucleic acid extraction methods for both fresh and herbarium plant and fungal specimens. In Part II, results are presented from two fungal taxonomic and phylogenetic studies that have relied mainly on herbarium specimens. In Part III, statements of future plans are presented.

PART I: A COMPARISON OF DNA EXTRACTION METHODS

INTRODUCTION

Historically, extraction of usable nucleic acids from plants and fungi has been difficult, in some instances notoriously so. Both plants and fungi produce polysaccharides, sometimes in copious amounts, that can cause problems in subsequent use of the DNA (with restriction enzymes, polymerases, etc.). Drying, lyophilization and mummification appear to have little effect on removal or inactivation of the inhibitors. Therefore, in order to extract the DNA for use in molecular biology experiments, removal of the offending compounds must be accomplished. In addition, nucleases and other enzymes must be removed.

Several steps are necessary in all nucleic acids extraction methods. First the cells must be broken, which is generally accomplished by grinding in dry ice or liquid nitrogen. In the cases of fossil, mummified and herbarium specimens, grinding can be done without freezing since the tissues are already sufficiently broken and brittle. Second, nucleases and other enzymes must be disabled and removed. This is accomplished through a combination of EDTA (to chelate Mg^{2+}, a cofactor for many nucleases), detergent (to disrupt membranes), heat, and buffer. CTAB (cetyltrimethylammonium bromide), SDS (sodium dodecyl sulfate) and Triton X-100 are the most often used detergents. In addition, most extraction methods include PVP (polyvinylpyrrolidone), DTT (dithiothreitol), or b-mercaptoethanol to inhibit polyphenol oxidase and other enzyme activities. Next, the proteins are further disabled and removed with either phenol and/or chloroform. As was stated, the polysaccharides must be removed, since some inhibit enzyme activity in later manipulations of the DNA. CTAB, which complexes with polysaccharides and with DNA (under different conditions) has been used often in plant and fungus nucleic acids extractions. Many polysaccharides are insoluble in the presence of CTAB and moderate (around 1 M) salt concentrations, while DNA and RNA remain in solution. The DNA can be precipitated by lowering the salt concentration of the solution or by addition of alcohol (isopropanol or ethanol). If a CTAB/DNA precipitate is obtained, the CTAB must be removed prior to use with enzymes. This can be done by addition of salt (normally NaCl) to a

concentration of about 0.5 - 1.0 M. The sodium salt of DNA can then be precipitated by addition of alcohol, leaving the CTAB in the supernatant.

Many methods employ the use of CsCl isopycnic gradients to remove both proteins and polysaccharides. However, inhibition of restriction and other enzymes is often encountered even after purification by CsCl gradients. Also, CsCl gradients are impractical when milligram and submilligram amounts of tissue specimens are used, since much of the DNA might be lost in the gradients. Therefore, for herbarium, mummified or fossil specimens, CsCl gradients are contraindicated. A major advantage of CTAB methods is that CsCl gradients can be completely eliminated, thus expanding their potential utility. Their widespread use is proof that they are valuable methods in plant and fungus nucleic acid extractions.

Several methods were utilized to determine the best method(s) for DNA extraction from plant and fungal tissues. It is crucial that a reliable method be chosen for herbarium, mummified and fossil specimens that produces adequate yields of readily usable DNA. While this is not an exhaustive list of methods, the attempt was made to choose methods that are successful with small specimens, avoid CsCl gradients, and are in wide use (also indicating successful extractions). Six basic methods were tested and compared, gathered from sixteen selected publications.

MATERIALS AND METHODS

Plant specimens (used for all methods): *Acer saccharum* (sugar maple) herbarium leaves (SYRF5160), *Citrus limon* f. (lemon) fresh leaves, *Coffea arabica* (coffee) fresh leaves, *Euphorbia pulcherrima* (poinsettia) fresh leaves, *Helianthus annuus* (sunflower) fresh embryos and cotyledons, *Pinus lambertiana* (sugar pine) fresh embryos, *Vicia faba* (broad bean) fresh embryos and cotyledons, *Vitis vinifera* (grape) preserved leaves, *Zea mays* (maize) fresh embryos.

Fungal specimens (used for all methods, all were herbarium basidiomata): *Agrocybe praecox* (SOR0005), *Coprinus comatus* (SOR0029), *Dermocybe marylandensis* (JFA7936), *D. marylandensis* (R.Hallings2331), *D. marylandensis* (DMS1995), *D.* sp. (YL1001), *Naematoloma capnoides* (SOR0114), *Psathyrella hydrophila* (MLS1000), *Suillus luteus* (MLS1001).

Extraction methods: The six basic methods are listed below, as "**Method A-F**." All fresh tissues were ground to a powder in microfuge tubes or mortars and pestles using dry ice. Tissue samples ranged from 15-100 mg, although the same amounts of each type of tissue were identical from one method to another. All herbarium tissues were ground in microfuge tubes without dry ice, using 5-25 mg of tissue. All were stored at -20°C until the extractions were performed.

Method A - For each milligram of powdered tissue, 1-2 ml of 65°C extraction buffer (2% CTAB, 100 mM Tris (pH 8.0), 20 mM EDTA, 1.4M NaCl and 2% polyvinylpyrrolidone (PVP) [M_r 40,000]) was added and mixed thoroughly. The mixture was then incubated at 65°C for 5-15 min. The mixture was emulsified with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged for 5 min in a microfuge. The aqueous phase was transferred into a new microfuge tube, followed by addition of a 5% CTAB/0.7M NaCl solution. A second chloroform/isoamyl extraction was then performed. The aqueous phase was transferred into a new microfuge tube and an equal volume of precipitation buffer (1%CTAB, 50 mM Tris (pH 8.0), 10 mM EDTA, 1% PVP) was added, followed by mixing with gentle inversion of the tube. The precipitate was pelleted in a microfuge for 30 sec to 5 min (depending on the amount of

precipitate). The pellet was rehydrated in 50-250 ml of high salt TE (10 mM Tris (pH 8.0), 1 mM EDTA, 1 M NaCl) by heating at 65°C for 5-15 min, and occasional vortexing. Two volumes of cold 95% ethanol were then added, followed by gentle mixing. Following centrifugation in a microfuge, the pellet was dried by vacuum and was rehydrated in 20-200 ml 0.1X TE (1 mM Tris (pH 8.0), 0.1mM EDTA) [This method was based on Murray and Thompson (1980) for fresh plant specimens, Taylor and Powell (1982) for fresh plant specimens, Rogers and Bendich (1985, 1988) for fresh, herbarium and mummified plant specimens, Rogers et al. (1989) for fresh and herbarium fungal specimens and Shivji et al. (1992) for marine macroalgae specimens. We have used the method to successfully extract DNA from algae, bacteria, fungi (ascomycetes, basidiomycetes, and deuteromycetes), insects, lichens, mammals, mycorrhizae, plants, reptiles and slime molds. The Murray and Thompson (1980) and Taylor and Powell (1982) methods used CsCl gradients, which were circumvented in the present experiments.]

Method B - The initial steps in this method are similar to those in method A (above). The extraction buffer was identical except 2% b-mercaptoethanol was included instead of PVP. Incubation was at 65°C for 30-60 min. Chloroform/octanol (24:1) was substituted for chloroform/isoamyl alcohol. The 5% CTAB/0.7M NaCl was not used in this method. Following the two chloroform/octanol extractions, 2/3 volume of isopropanol was added, followed by gentle mixing. The precipitate was pelleted by centrifugation. Since we occasionally encountered problems of inhibition in digestion with enzymes, the following additions to the procedure were made. The pellet was rehydrated with high salt TE (as above) and was precipitated with ethanol, followed by centrifugation, drying and rehydration in 0.1X TE. [This method was based on Saghai-Maroof et al. (1984) for fresh plant specimens, Doyle and Doyle (1987) for fresh plant specimens and Zolan and Pukkila (1986) for fresh fungal specimens.]

Method C - For each milligram of tissue 1-2 ml of extraction buffer (20 mM Tris, 100 mM EDTA, 300 mM NaCl, 50 mg/ml proteinase K) was added. The mixture was incubated at 4°C for 15 min. SDS was added to a total concentration of 2%, and the mixture was incubated at 65°C for 30-40 min. Next, the NaCl concentration was increased to 1.4 M, then 1/10 volume of a 10% CTAB solution (10% CTAB, 100 mM Tris (pH 8.0), 100 mM EDTA) was added. The mixture was incubated at 65°C for 10 min, then 15°C for 2 min. Two chloroform/isoamyl extractions were performed, followed by ethanol precipitation (as above) and drying, then rehydration in 0.1X TE. [This method is based on Kim et al. (1990) for fungal specimens and Möller et al. (1992) for fungal and plant specimens.]

Method D - For each milligram of tissue 1-2 ml of extraction buffer (200 mM Tris (pH 8.0), 100 mM EDTA, 1 M NaCl, 20 mM b-mercaptoethanol, 2.5% SDS) was added. The mixture was incubated at 65°C for 10 min. Two chloroform/isoamyl alcohol extractions were then performed. The aqueous phase was then precipitated by addition of 1/10 volume of a spermine buffer (100 mM Tris (pH 7.0), 100 mM spermine), and placing on ice for 15 min. After centrifugation for 5 min, the pellet was rehydrated in high salt TE (as above), followed by ethanol precipitation, drying and rehydration in 0.1X TE. [The initial steps in the procedure are based on Dellaporta et al. (1983), while the spermine precipitin steps are based on Hoopes and McClure (1981).]

Method E - For each milligram of tissue, 1-2 ml of extraction buffer (400 mM Tris (pH 8.5), 600 mM LiCl, 20 mM EDTA, 2% deoxycholic acid, 2 mM aurintricarboxylic acid, 10 mM thiourea, 3.0% lithium dodecyl sulfate, 2% Nonidet P-40,

20 mM DTT) was added. The mixture was incubated at 4°C for 30 min. This was followed by two extractions with chloroform/isoamyl alcohol. The aqueous layer was then precipitated with ethanol, dried and rehydrated in 0.1X TE (as above). [This method was based on Baker et al. (1990) for plant specimens, and was originally used in conjunction with CsCl gradients, which were circumvented here.]

Method F - For each milligram of tissue, 1-2 ml of extraction buffer (2% SDS, 300 mM NaCl, 200 mM Tris (pH 8.0), 20 mM EDTA, 100 mM DTT, 1.0 mg/ml proteinase K) was added. The mixture was incubated at 37°C for 1 h, followed by two chloroform/isoamyl alcohol extractions. One-tenth volume of 3M sodium acetate was added, followed by ethanol precipitation, drying and rehydration in 0.1X TE (as above). [This method is based on Pallsboll et al. (1992) and Kocher et al. (1989), both for animal specimens. The original procedures call for chloroform/phenol extractions rather than the chloroform/isoamyl alcohol extractions used here.]

DNA/RNA assays: Digestions with *Eco*RI and RNase were as in Rogers and Bendich (1985). PCR (polymerase chain reaction) amplifications were as in Yan et al. (1993a,b). The conditions of the DNA and RNA were determined by subjecting the nucleic acid solution to electrophoresis on 0.6% agarose gels. The lanes of DNA were observed on the photographic negative for high molecular weight DNA (>50 kb) as well as any distribution of lower molecular weight DNA. The DNA was then categorized as high molecular weight (i.e., no degradation), somewhat degraded or highly degraded. The 25S and 18S rRNA bands were either very sharp, smeared or absent. The RNA condition was thus categorized into the same categories as DNA (above). The DNA yields were based on measurement of an aliquot of the DNA viewed on agarose gels compared to known amounts of lambda (l) DNA. Color of the nucleic acid solutions was categorized as light (clear), moderately colored (clear yet obviously colored) or darkly colored (opaque). Optical density spectral measurements at 230 nm, 260 nm and 280 nm were also performed.

RESULTS AND DISCUSSION

The results are summarized in Table 1. Comparative rankings for the six methods are presented as well as portions of the quantitative and qualitative results. For method A, while yield was moderate compared to the other five methods, the condition of the DNA was generally good and little or no inhibition of subsequently added enzymes was evident. The results from method B were similar to those for method A, although the degree of degradation of DNA and RNA was greater. For method C, the yield and condition of the DNA and RNA were the best of all of the methods. However, the DNA solutions were often dark (indicating polyphenol oxidase activity) and frequent inhibition of *Eco*RI and RNase was observed. For method D, the yield and condition of the DNA and RNA were poor. Inhibition of *Eco*RI and RNase was also evident in some samples. For method E, the yield and condition of the DNA were good, except the coloration of the DNA solutions was often very dark. Additionally, nearly complete inhibition of *Eco*RI and RNase was observed in most nucleic acid solutions. Method F produced average results for almost all assays.

Spectral absorbance ratios ($A_{260/280}$ and $A_{260/230}$) did not correlate with utility (digestion with enzymes and PCR amplification) of the DNA, thus the ratios were not included in Table 1. Storage tissues (e.g., cotyledons) often contained a great deal of polysaccharide materials that were not efficiently removed by any method. However,

Table 1. DNA extractions results. For each cell in the table, the top number (in bold) indicates the rank of the method compared to the other methods (1=best). The lower numbers are qualitative and quantitative results from the assays of the methods. All data were from extractions and assays from the 20 specimens listed in the materials and methods section. Methods are as follows (see also Materials and Methods): **A** = Murray and Thompson (1980); Rogers and Bendich (1985, 1988); Rogers et al. (1989); Shivji et al. (1992); Taylor and Powell (1982); **B** = Saghai-Maroof et al. (1984); Doyle and Doyle (1987); Zolan and Pukkila (1986); **C** = Kim et al. (1990); Moller et al. (1992); **D** = Dellaporta et al. (1983) plus Hoopes and McClure (1981); **E** = Baker et al. (1990, without CsCl); F = Pallsboll et al. (1992).

	A	B	C	D	E	F
DIGESTIONS[1]	**1**	**1**	**3**	**2**	**4**	**3**
EcoRI(complete/partial/inhibition)	(18/2/0)	(20/0/0)	(10/9/1)	(16/3/1)	(1/10/9)	(10/6/4)
RNase(complete/partial/inhibition)	(20/0/0)	(20/0/0)	(16/4/0)	(19/1/0)	(2/18/0)	(15/5/0)
PCR AMPLIFICATION[2]	**1**	**1**	**2**	**1**	**3**	**2**
	(+)	(+)	(+/-)	(+)	(-)	(+/-)
DNA/RNA CONDITION[3]	**2**	**3**	**1**	**3**	**1**	**2**
	(72/32)	(55/29)	(89/50)	(65/10)	(100/33)	(78/44)
YIELD All Tissue Types[4]	**3**	**2**	**1**	**4**	**2**	**2**
	(40)	(59)	(80)	(31)	(59)	(51)
Fresh Tissues	**4**	**1**	**1**	**5**	**2**	**3**
	(31)	(88)	(96)	(17)	(58)	(45)
Herbarium Tissues	**2**	**3**	**1**	**2**	**1**	**1**
	(43)	(29)	(62)	(45)	(59)	(58)
COLOR[5]	**1**	**1**	**3**	**2**	**4**	**2**
	(L)	(L)	(M)	(L-M)	(M-D)	(L-M)
TIME OF PREPARATION[6]	**1**	**1**	**3**	**1**	**2**	**1**
(MEAN)	3.5 h	3.5 h	4.5 h	3.5 h	4.0 h	3.5 h

[1]Based on complete/partial digestion/complete inhibition of the enzymes.
[2]Based on amplification with primers ITS3 and ITS4 (White et al., 1990).
[3]Based on high molecular weight DNA band and 25S, 18S rRNA and tRNA bands on 0.6% agarose gels. Numbers in parentheses are: (percentage of extractions with high molecular weight DNA/percentage of extractions exhibiting RNA bands).
[4]Numbers in parentheses are the mean yields in nanograms of DNA per milligram of starting tissue.
[5]Color of DNA solution indicating polyphenol oxidase (and other enzymes) activity during extraction. In parentheses, L = generally clear or faintly colored, M = moderate coloration, D = dark or opaque.
[6]Based on simultaneous extraction of DNA from all 20 specimens.

the remaining contaminants (including polysaccharides) appear to be unimportant to the utility of the resultant nucleic acid solutions.

In general, success in DNA extraction is measured in DNA yield, condition (molecular weight and color), and utility (or ease of use with restriction enzymes, polymerases, ligases, etc.). While yield is somewhat important, especially when milligram and submilligram amounts of tissue are used, it is less important than the condition of the DNA. The methods utilizing CTAB (methods A, B, and C) produced DNAs generally with less inhibition of enzymes (except method C). While the yields were lower than for some of the other methods, the yields were still adequate for most molecular biology uses. Both method A and B (Table 1) produced DNA that was always digested with *Eco*RI and RNases. This was true even when large amounts of the DNA solutions (up to 85% of the total volume) were introduced into the restriction enzyme reaction mixture. The condition of the DNA was also good to excellent. The highest yields were from one of the CTAB methods (method C). The lack of significant degradation of both DNA and RNA was initially encouraging. However, significant amounts of contaminants were carried with the nucleic acids such that both *Eco*RI and RNases were often inhibited. Dilution in the PCR reactions, up to 1:20 failed to remove the inhibitory effects. Inhibition of enzymes in the C and E methods could be due to carryover of nuclease inhibitors added during extraction. Although methods C and E appear to be best in terms of yield and nucleic acid condition, further purification would be required to eliminate enzyme inhibitors. Some of the methods examined suggest the use of CsCl gradients, which can further purify the DNA away from potential inhibitors. Further alcohol precipitation might also be used to eliminate the inhibitors. However, these methods already require more time for preparation and extraction than any of the other methods. In general, methods A and B are recommended for most purposes, and especially recommended for herbarium, mummified and fossil specimens.

PART II: FUNGAL TAXONOMY AND PHYLOGENETICS

A. MOLECULAR DATA IN FUNGAL SYSTEMATICS AND PHYLOGENETICS

Molecular analyses have been utilized in fungal taxonomic and phylogenetic studies with increasing frequency. Although plants and other higher eukaryotes exhibit many useful morphological, ecological, biochemical and other characters, fungi exhibit fewer distinguishing characters. Additionally, the characters may not be continually exhibited. In some cases where few morphological characters are available (e.g., fungi, especially deuteromycetes, where there is no sexual stage) molecular characters are invaluable. DNA characters are direct indicators of genetic identity when the appropriate sections of the genome are analyzed. Restriction length polymorphisms (RFLPs), restriction site maps and DNA sequences have been used in numerous systematic studies (Anderson et al., 1987, 1990; Anderson and Petsche, 1986; Anderson and Smith, 1989; Bruns et al., 1989, 1991; Bruns and Palmer, 1989; Gardes et al., 1990; Harney et al., 1992; Kohn, 1992; Liu et al., 1992; LoBuglio et al., 1991; Nelson et al., 1990; Rogers et al., 1989; Shinohara et al., 1992; White et al., 1990; Yan et al., 1992, 1993a,b).

RFLP analyses have been employed in a number of fungal studies to confirm or establish rough limits for specific taxa (Anderson and Petsche, 1986; Anderson et al., 1987, 1990; Bruns et al., 1989, 1991; Rogers et al., 1989; and our unpublished data). We used this type of analysis to examine the ribosomal DNA (rDNA) from isolates of the deuteromycete *Cenococcum geophilum*, and determined that multiple species and

probably multiple genera are included in this *form species* (LoBuglio et al., 1991). In our studies of basidomycetes, some species of the morphologically heterogeneous genus *Inocybe* exhibited more than one rDNA repeat type, indicating either a mixed rDNA locus, more than one locus, or more than one species (unpublished data). At the other extreme, we found that four distinct species of *Amanita* exhibited the same rDNA hybridization patterns for four enzymes, indicating a genetically homogeneous rDNA in this genus. Clearly, rDNA characterizations at this level are insufficient to satisfactorily delineate these (and many other) taxa, but they can be used to make initial groupings of many taxa.

Fine structure restriction enzyme mapping to produce high resolution maps can often provide more useful taxonomic and phylogenetic information than can the low resolution maps. Relatively short regions (generally less than 2 kb) can be amplified using PCR and then mapped with a dozen or more restriction enzymes. A fairly large and informative data set can be generated in a short time. While this method is usually more informative than RFLP analyses of the entire rDNA region, the resolution is insufficient to answer many taxonomic questions. However, it is excellent for grouping similar or identical isolates preceding the initiation of DNA sequencing.

DNA sequence analysis is probably the best method for delineating taxa. Each base pair can be used either for sequence alignment or for taxa comparisons. Because the data sets are generally large, very fine relationships can usually be determined. However, sequencing is expensive and time consuming, both in the lab and during analysis. Therefore, usually only a subset of specimens are sequenced. Initial screening by RFLP analysis, mapping and fine structure mapping can aid to lessen the number of isolates on which to perform DNA sequence analysis and to identify an appropriate region of the genome (Figure 1). Another benefit of sequencing is in the development of tools for identification. From DNA sequence comparisons, regions that are taxon-specific (at many levels) can be identified. These can then be used to generate specific probes for hybridization analyses or primers for PCR amplifications, both of which can be used to rapidly identify particular unknown specimens.

For all molecular biology investigations, since only a relatively small region is analyzed, great care must be exercised in selection of an area to sequence, and it is always desirable to focus on a well-characterized DNA region. Also, probes or primer regions are more accessible when a known gene region is used. Generally, a region containing at least part of one gene is chosen, since genic regions vary less than nongenic regions, thus simplifying data alignment. Since various regions of the genome (including genes) change at different rates from one organism to the next it is critical to choose the appropriate region for the question posed (see e.g., Doyle, 1992; Kohn, 1992). Depending on the methods and regions used, distinctions can be made at virtually any chosen level, from variation within populations up to differences between Kingdoms.

The areas most often analyzed have been mitochondrial (mtDNA) and nuclear ribosomal (rDNA). Comparisons of mtDNA have been made in taxonomic and phylogenetic studies for a number of fungi, including, *Agrocybe* (Rehner, 1989), *Armillaria* (Anderson et al., 1987), *Exophiala* (Kawasaki et al., 1990) and *Suillus* (Bruns and Palmer, 1989; Bruns et al., 1988, 1989). However, mtDNA characters must be analyzed cautiously, since mitochondrial genomes can change rapidly, especially in plants and fungi (Bendich, 1985; Bruns and Palmer, 1989; Bruns et al., 1988, 1989; LoBuglio et al., 1991; Palmer and Hebron, 1987; Shumard et al., 1986; Toothman et al., 1988). Above the varietal and species levels, mtDNA is generally not useful because

alignments of the sequences and maps are difficult due to high levels of variability. Additionally, mixtures of heterogeneous mitochondrial genomes can exist within populations and within single organisms. This severely limits the use of organellar genome data in taxonomic and phylogenetic investigations. Since exchange of mtDNA

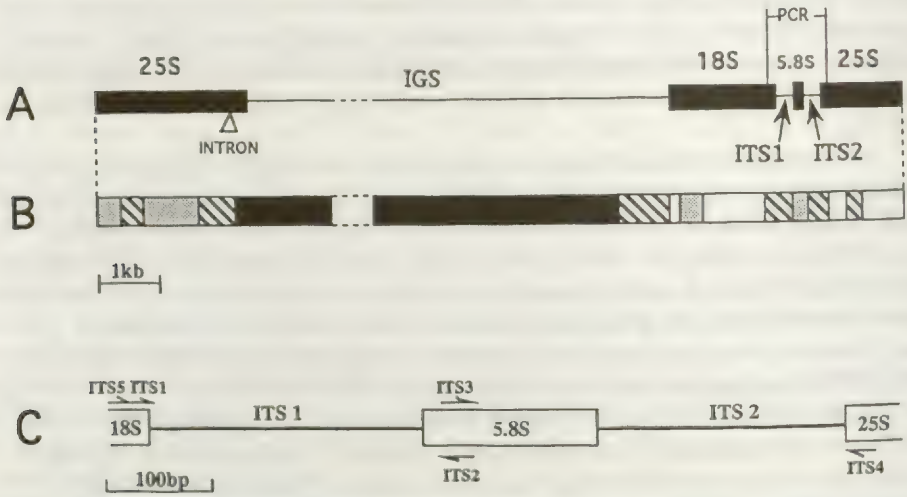


Figure 1. Ribosomal DNA regions in fungi. **A.** Diagram of one rDNA repeat unit. IGS = intergenic spacer, ITS = internal transcribed spacer. Dashed lines of the IGS indicate the variable length of the IGS. **B.** Variability map of the rDNA region (as in A) based on restriction enzyme maps of four enzymes (*Cla*I, *Eco*RI, *Stu*I, and *Xba*I) for 75 species (from 21 families) of basidiomycetes (unpublished data). Dark regions are highly variable, striped regions are variable, stippled regions are slightly variable, and lightest regions are highly conserved. **C.** Diagram indicating the region analyzed by PCR (see A, above), fine structure mapping and sequencing. It contains variable regions (ITS1 and ITS2) as well as more conserved regions (the ends of the 18S and 25S genes and the 5.8S gene). Primers used for PCR amplification are indicated by the darts with designations of ITS1 - ITS5 (from White et al., 1990).

is possible during parts of fungal life cycles, mtDNA transfer, hybridization, recombination, dilution and/or complete loss of one (or more) of the genomes can cause confusion in the interpretation based on slow methodical DNA changes. It has been suggested that organellar genomes be considered separate organisms, and should not be used to indicate the taxonomy or phylogenetics of the corresponding nuclear genome, unless substantial accompanying data supports these conclusions (Doyle, 1992).

Ribosomal DNA has been used in a wide variety of taxonomic and phylogenetic comparisons. Because of this, sequence data, clones of rDNA for probes, and PCR primers are readily available from a variety of sources. These genes exist in multiple copies so that the signal from hybridized probe DNA is easily detectable, and PCR amplification is easily accomplished, in unfractionated total DNA samples (Harney et al., 1992; John and Miklos, 1988; Lewin, 1990; Liu et al., 1992; Long and Dawid, 1980; Rogers and Bendich, 1987; Shinohara et al., 1992; Yan et al., 1992, 1993a,b). Although they exist in multiple copies, they are inherited essentially as a single version, since gene conversion rates are high in this region. This is unlike many other multicopy genes and gene family traits where many versions may exist within the same genome. They have

been extensively studied and characterized in most eukaryotic groups, thus facilitating further study and comparison (Anderson et al., 1987, 1990; Borsuk et al., 1982; Cassidy et al., 1984; Free et al., 1979; LoBuglio et al., 1991; Lockington et al., 1982; Rogers and Bendich, 1987, 1988; Rogers et al., 1989; Russell et al., 1984; Specht, et al., 1984). Much of the intergenic spacer (IGS, also known as the nontranscribed spacer, or NTS; Figure 1) changes rapidly even among closely related taxa. Parts of the internal transcribed spacers (ITS1 and ITS2; Figure 1) and the genic regions change at moderate to high rates, while other portions of the genic regions are highly conserved (Appels and Dvorak, 1982; Reeder, 1985; Rogers and Bendich 1987; Rogers et al., 1989). Therefore, depending on the questions posed, the appropriate regions can be compared between taxa to determine their relationships.

For most of our research, we have concentrated on the region from the 3' end of the 18S nuclear rRNA gene to the 5' end of the 25S gene (Figure 1). This contains three gene regions (the ends of the 18S and 25S genes and the entire 5.8S gene) for restriction map and sequence alignment, and for comparison of distantly related isolates. It also includes two spacer regions (ITS1 and ITS2) for comparison of closely related isolates. We are also initiating study of two other gene regions, histone genes H3 and H4, and the actin gene regions. Each contains conserved as well as variable regions. Both are well-characterized in the literature for a variety of organisms, so that primers can be constructed and sequences can be compared (John and Miklos, 1988).

B. Phialophora - A DEUTEROMYCETE

Fungi reproduce both sexually and asexually, but are classified on the basis of sexual reproductive structures, i.e., the teleomorphs. The Subdivision Deuteromycotina (deuteromycetes, imperfect fungi) encompasses all the fungi whose sexual stages are unrecognized or are absent. These fungi reproduce asexually by forming conidia (spores) or by budding. Since there are no teleomorphs, these fungi are all designated as *form species* within *form genera*. This Subdivision includes approximately 1700 *form genera* and 17,000 *form species* (Hawksworth et al., 1983). Some deuteromycetes have been found to be allied with ascomycetes, representing the anamorphic and teleomorphic stages, respectively. However, the majority of the deuteromycetes have no known sexual relative. Therefore, classification of these fungi is based solely on the morphological and some physiological or biochemical characters of the asexual, conidial stage (the anamorph). Compared to most other organisms, the number of useful taxonomic characters in deuteromycetes is very small. Compounding these deficiencies is the fact that many are pleomorphic, that is, they have more than one spore-producing form. There is a great need to find other useful characters for this diverse group of fungi. The problem of accurate determination becomes critical in the view of the importance of deuteromycetes in the environment, industry, and human medicine (Rippon, 1988; Wang and Zabel, 1990). These cultures are handled routinely by laboratory technicians who may not be aware of the potential pathogenicity in humans. Infections have become more prevalent recently since they often infect immunosuppressed patients.

The form genus *Phialophora* was erected for the single species *P. verrucosa* Medlar based on an isolate from human dermatosis (Medlar, 1915). The species was characterized by an ampulla-form asexual reproduction structure (the phialide) with a "small shallow cup" (the collarette). Subsequently, the genus *Cadophora,* with the type species *C. fastigiata* that caused stains in softwood timber, was proposed (Lagerberg et

al., 1927). Melin and Nannfeldt (1934) added five new species of *Cadophora* and Davidson (1935) described two more species that were all isolated from stained wood or pulpwood products. Eventually, *Phialophora* and *Cadophora* were combined and the eight species of *Cadophora* were transferred into *Phialophora* (Conant, 1937). The species *P. americana* was proposed as a split from *P. verrucosa* (Hughes, 1958), although this has not been universally accepted. Most isolates of *P. americana* have originated from plant and soil sources whereas those of *P. verrucosa* have come mainly from human mycoses. The morphological differences of these isolates vary from subtle to considerable. One of the major distinguishing characters is that *P. americana* forms deeper collarettes on the phialides than does *P. verrucosa*.

Dried and fresh cultures from known sources used in this study were of importance in two ways. First, herbarium specimens are invaluable in studies of deuteromycetes, and fungi in general, especially those that are difficult to culture and identify. For example, some of the species examined require 1-2 years in cold culture to induce sporulation for morphological identification. Second, some specimens sent to us were misclassified, thus alerting us to the potential for significant errors in data sets. For example, the two isolates in group 8 (Figure 2) were sent to us as "*P. verrucosa*" which they clearly are not. Because of this, caution should be used when interpreting results extracted from distant sources. Identification should be confirmed by experts who are familiar with the specific fungus being utilized. The names written on voucher specimens may sometimes be incorrect.

The primary objective of this study was to delineate *P. verrucosa* and *P. americana* employing both molecular and morphological characters. Fine structure analysis of the 5.8S gene and ITS rDNA regions using PCR and restriction mapping for systematics shows promise, although the paucity of published accounts make complete evaluation difficult. Based on the rDNA restriction data we determined that there were at least seven restriction map groups within the *P. americana/P. verrucosa* complex (Figure 2; Yan et al., 1993a). We also wanted to determine the relationships between the *P. verrucosa/P. americana* complex and two other species, *P. richardsiae* and *P. parasitica*. Isolates representing *P. richardsiae* and *P. parasitica* increased the number of restriction map groups to thirteen. Many differences were observed between the *P. americana/P. verrucosa* complex and the other groups, while fewer differences were evident among isolates of *P. americana* and *P. verrucosa* (Yan et al., 1993a). With the addition of morphological data, a more definite demarcation between the two species was demonstrated.

The rDNA data were consistent with morphological characters as well as total protein profiles of *P. americana* and *P. verrucosa* (Zweibel and Wang, 1977). The trees produced from the restriction maps have shown separation of 29 *P. americana/P. verrucosa* isolates into 6 phenotypic groups (groups 1, 2, 3, 5, 6, and 7) and into two clusters corresponding to the two morphological species *P. americana* and *P. verrucosa*. However, group 4 with three isolates was placed between the two clusters, although morphologically the three isolates were closer to *P. verrucosa*. This result indicates the resolution limitations of restriction analysis of this region of rDNA for closely related species. [Restriction mapping also indicated the presence of a short insert near the 3'end of the 18S rRNA gene. Sequencing and other data confirmed that a 67 bp intron existed near the 3' end of the 18S gene in the two isolates in group 7. Except for 2 bp differences, the two were otherwise identical. This intron (described in Rogers et al., 1993) has characters in common with messenger RNA-type introns.]

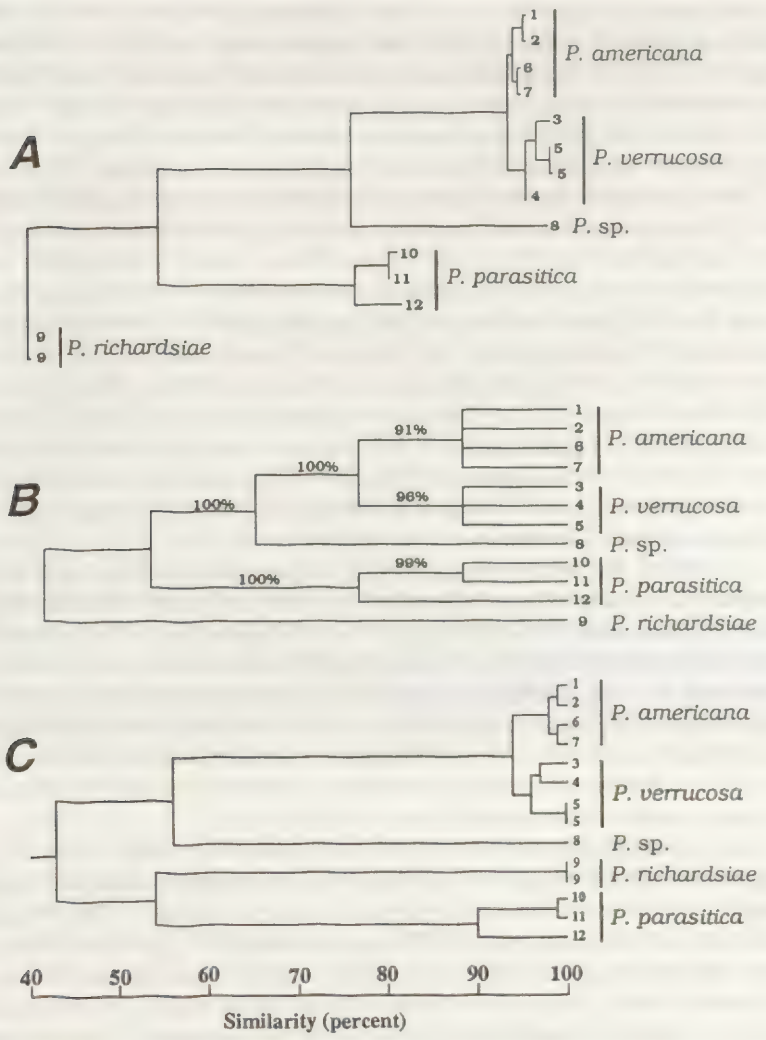


Figure 2. Phylograms and phenogram of the relationships within *Phialophora* based on sequence data. **A.** Phylogram produced using PAUP. Only one most parsimonious tree was produced from the data. Numbers represent the restriction map groups. **B.** Bootstrap phylogram from PAUP analysis of the same data as in A, from a search of 1000 trees. Percentages indicate the frequency that the branch was identical in the search. **C.** Phenogram using NTSYS (Rohlf, 1992) produced from the same data as above.

Phylogenetic (PAUP, Swofford 1990) and phenetic (NTSYS, Rohlf, 1992) analyses of sequence data indicated a separation of isolates of *P. americana* from isolates of *P. verrucosa* (Figure 2). This is consistent with morphological and protein profile data (Zweibel and Wang, 1977). Restriction and sequencing data also indicate some variation within some species. However, these genetic variations do not reflect any significant morphological changes. Except for the low rate of change exhibited between *P. americana* and *P. verrucosa*, variations were high in both ITS1 and ITS2 regions

between species but relatively low within each species (Yan et al., 1993b). The gene regions were highly conserved within each species and generally within the genus. The changes in the 18S, 5.8S, and 25S genes indicated a splitting of the five *Phialophora* species into two groups (*P. americana*, *P. verrucosa*, and *P.* sp. (group 8) were in one group, while *P. richardsiae* and *P. parasitica* were in the other group). Cluster analyses (Figure 2C) placed isolates of *P. americana* together at similarities of 98-99%. Isolates of *P. verrucosa* were clustered together at similarities of 96-100%. The *P. americana* and *P. verrucosa* clusters were very close with 94% similarity. Group 12 of *P. parasitica* exhibited only 90% similarity with the main group of this species. Phylogenetic analyses, always placed *P. verrucosa* close to *P. americana*, but on separate branches. The bootstrap analysis strongly separated the isolates of *P. americana* and *P. verrucosa* into two species. This statistical analysis provides strong support in splitting this species complex (Mitchell et al., 1992). After comparing the rDNA data with morphological characters and total protein profiles (Zweibel and Wang, 1977) it appears that *P. americana* and *P. verrucosa* are two distinct but closely related species. The two non-sporulating isolates that were originally designated as *P. verrucosa* (group 8, Figure 2) should now be placed into a separate group, and might represent an undescribed species.

Phialophora parasitica was found to be genetically heterogeneous based on restriction and sequence data. Sequence comparison indicated a relatively large distance (36 bp) between one isolate and the type strain of *P. parasitica*. This distance was greater than the distances between all isolates of *P. americana* and *P. verrucosa*. Weitzman et al. (1984) studied the morphology, physiology and antifungal sensitivity of several isolates of *P. parasitica* (including all of the isolates in this study). They suggested that the identity of some isolates was uncertain, but the one in question used in this study fell within the range of this species. Additional morphological study is in progress to elucidate the species status and limits.

As expected, the analyses have indicated generally that the 5.8S gene region is conserved and the ITS regions are variable. The 5.8S gene has sufficient sequence change for genus and species level systematic studies. Variation in the 5.8S gene was found to be low and variation in the ITS regions was high. Sequence differences between the species in this study varied significantly from a minimum of 13 bp between *P. americana* and *P. verrucosa* to a maximum of 196 bp between *P. verrucosa* and *P. parasitica*. Except for the differences between *P. americana* and *P. verrucosa*, the differences between any two species (including the non-sporulating isolates which are now considered as unknown species of *Phialophora*) were from 131 bp to 196 bp. These differences probably accurately indicate the genetic distance of these species, although other gene regions must be examined to show this conclusively. Classification of deuteromycetes based on the asexual reproducing structures might not represent the biological relationships among this diverse group of fungi. Molecular studies of the species in deuteromycetes, especially of those species where the sexual stage may have been lost permanently, can provide valuable genetic information that could lead to a thorough understanding of the relationships of these fungi.

C. *Cortinarius* and *Dermocybe* - BASIDIOMYCETES

Definitive taxonomic treatments for *Dermocybe*, *Cortinarius* and several other ecologically important ectomycorrhizal agaric genera are unavailable for North America. This is due primarily to the large sizes and complexities of these genera, making the

circumscription of taxa very difficult. In addition, the extensive distributions of many of the taxa make the solutions to taxonomic problems in these genera challenging. These ectomycorrhizal fungi are difficult to isolate and maintain in culture and their basidiospores cannot be readily germinated to produce monokaryons (haplotypes). Therefore, crossing studies to establish lines of compatibility cannot be done. As a result, it is normally impossible to apply the biological species concepts. Therefore, taxonomy and phylogenetics of these fungi is done using characteristics such as morphology, pigmentation, anatomy and ecology, for which we have little or no genetic information. There is an extensive data base (of morphological, ecological, anatomical and biochemical characters) available for the subgenus *Dermocybe* in North America. Molecular data can provide valuable genetically based information which, when combined with other characteristics of these fungi, can allow definition of the taxa and phylogenetic relationships within and between taxa.

Species of *Dermocybe* produce small to medium sized basidiocarps (mushrooms) that have yellow, green, olive, yellow-brown, orange or red to purple-red lammellae (gills) and similarly colored stipes. The pilei (caps) may be identical in color with the lamellae and/or stipe, or browner in color. The coloration of the basidiocarps is due to the presence of a variety of intracellular and intercellular anthraquinonic pigments. The species form ectomycorrhizae and ectendomycorrhizae with certain trees and woody plants or perennials. *Dermocybe* species may be associated with a particular genus of plant or with several different genera. Some are associated with hardwoods, others with conifers. Many of the species are widely distributed. Investigators currently studying *Dermocybe* classify it either as a genus in the family Cortinariaceae, order *Agaricales* (Ammirati, 1988; 1989a; 1989b; Arnold et al., 1987; Keller, 1979; 1982; Moser, 1986b), or as a subgenus of *Cortinarius* (Høiland, 1984; 1985).

Moser (1983) lists 24 species for the *Dermocybe* in Europe. Høiland (1984) has reported 20 species in Nordic countries. There have been at least 40 species recorded from North America (excluding Mexico). *Dermocybes* in Europe and North America have been studied intensively (Ammirati, 1972, 1988, 1989a,b; Ammirati and Gilliam, 1975; Ammirati and Smith, 1969, 1977, 1978, 1984; Høiland, 1984, 1985; Moser, 1955, 1967, 1972, 1974a,b; 1978, 1983, 1986a; Orton, 1958; Robar et al., 1981). This has brought about several significant advances in the taxonomy of this group. Firstly, the species concepts for European taxa, especially those based on early names, has improved greatly. Secondly, the *Dermocybes* in North America and Europe have been more carefully studied than in the past, especially their pigmentation. Thirdly, we now know that many *Dermocybe* species occur both in Europe and in North America. Finally, more than two decades of work has resulted in a number of new taxa being named. It is becoming clear that there are a number of duplicated names, especially for widely distributed taxa. Molecular approaches combined with the other data sets may resolve many of the existing problems and questions. Within *Dermocybe* Moser (1986b) recognizes the sections *Dermocybe*, *Holoxanthae*, *Malicoriae* and *Sanguinea*. Høiland (1984) classifies all *Dermocybes* in the genus *Cortinarius*.

Comparison of the results from the molecular biology approaches with the results from biochemical (pigment), descriptive and ecological characteristics, have made possible taxonomic distinctions and phylogenetic relationships at very fine levels (Figures 4 and 5). For example, restriction mapping and sequencing of the ITS and 5.8S rDNA regions has indicated that *Dermocybe* is probably a genus distinct from the genus *Cortinarius*. Also, while current taxonomic groupings appear to agree well with the

phylogenetic analyses of molecular data (Figure 4), some reassessments are necessary. Comparisons of the groupings derived from the different data sets have been made to determine the consistency between the different types of data (morphological vs. molecular, etc.). Høiland (1984) used UPGMA analyses in his treatment of *Dermocybe* from Nordic countries (Figure 5). From the dendrograms he was able to generate a classification scheme for *Dermocybe* species, which differs in several ways from the

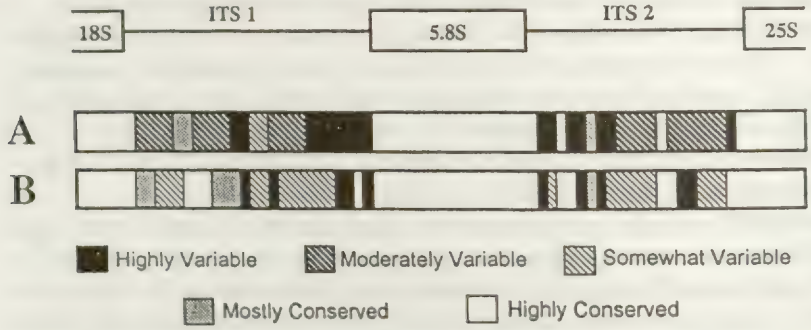


Figure 3. Variability maps of the ITS and 5.8S rDNA regions as determined from sequencing data of 18 specimens of *Dermocybe* and 2 specimens of *Cortinarius*. **A.** Relative variability in the rDNA regions between *Dermocybe* and *Cortinarius*. **B.** Relative variability in the rDNA region among specimens of *Dermocybe*. In general, more variability was observed between *Dermocybe* and *Cortinarius* than among the species of *Dermocybe* (Liu et al., 1992).

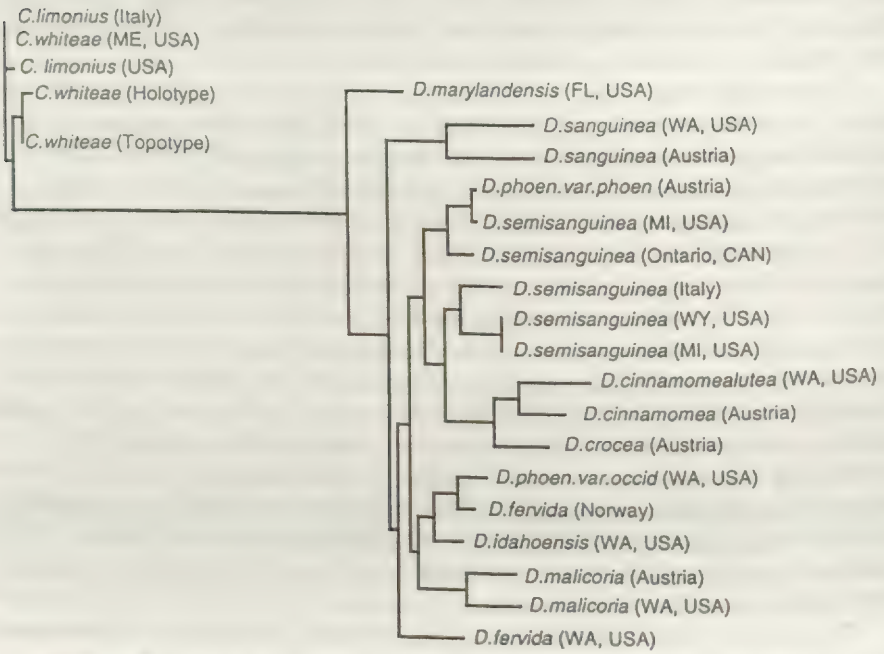


Figure 4. Phylogram of *Dermocybe* and *Cortinarius* determined from sequence data of the ITS and 5.8S rDNA regions using PAUP. Four equally parsimonious trees were obtained from the data. The one shown differs from the others in rotation of the branches and not distances between specimens (Liu et al., 1992).

traditional groupings indicated by Moser (1986b). Similarly, we have compared his analysis of morphological, ecological and biochemical characters (Figure 5) with our analysis of molecular characters (Figure 4). The two analyses come to similar conclusions in many cases, but differ significantly in other cases.

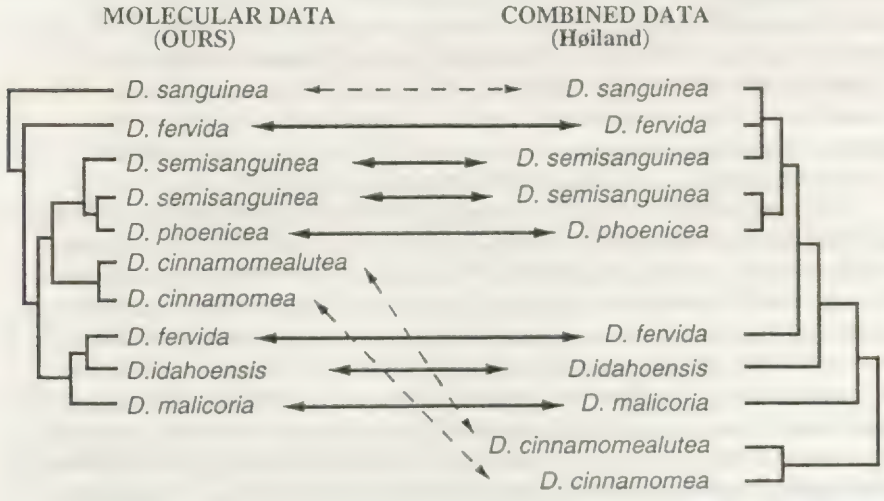


Figure 5. Comparison of our phylogram based on molecular characters, left (Liu et al., 1992), with the phenogram of Høiland (1984), right, based on a combination of data (morphological, ecological, and biochemical).

The main conclusions of this study are enumerated below:

1. The genus *Dermocybe* is clearly separated from the genus *Cortinarius* (Figure 4).
2. Specimens of *Cortinarius whiteae* and *C. limonius* appear to represent a single species (Figure 4).
3. *D. marylandensis*, considered by some to be a variant of *D. sanguinea*, appears to be a separate species (Figure 4).
4. *D. semisanguinea*, *D. phoenicea* var. *phoenicea*, *D. cinnamomea*, *D. cinnamomealutea* and *D. crocea* formed one large coherent cluster (Figure 4). This indicates the close relationship of these species.
5. Of the species where several specimens of that species were assayed, *D. semisanguinea*, *D. malicoria* and *D. sanguinea* exhibited tight species clusters, while specimens of *D. phoenicea* and *D. fervida* were often separated on the phylograms (Figure 4). This indicates that some species delimitations are inaccurate or the species are genetically variable. In either case, further investigation is required.
6. The European specimen of *D. phoenicea* was closest to North American *D. semisanguinea*, while the North American *D. phoenicea* was closest to the North American *D. fervida* (Figure 4) The European *D. fervida* was closest to *D. malicoria*. Clearly reorganization of these species might be needed.
7. Our data agree fairly well with the data of Høiland (1984). However, there are several differences (Figure 5). First, our data indicate that *D.*

sanguinea is distinctly separated from the other species, while Høiland places *D. sanguinea* closer to *D. semisanguinea*, *D. phoenicia* and *D. fervida*. Second, our data indicate that the *D. cinnamomea/D. malicoria* group is closer to *D. semisanguinea* than does his. Finally, our molecular data indicate that *D. cinnamomealutea* is not as closely related to *D. crocea* (only 80% similar) as Høiland finds the two (over 90% similar). We believe that much of the lack of resolution in parts of Høiland's analyses reflects the high degree of variability in the biochemically and ecologically based data, making them unreliable at this taxonomic level. The DNA-based comparisons appear to more accurately reflect the genetic relatedness of the taxa in *Dermocybe*, and in other fungal genera as well.

This study relied exclusively on the use of herbarium specimens. Most of the descriptive work for the North American species of *Dermocybe* has been completed; general collecting is no longer necessary. Therefore, the use of herbarium specimens greatly facilitated our work. If we had relied on fresh collections, many more years and decades, and a great deal of travel would have been necessary to complete this work. As it is, we will soon complete the study after approximately three years. The work on *Cortinarius* could only be done with herbarium tissues, since we were attempting to elucidate the correct identity of the species, and needed to use the holotype specimens for comparison with contemporary specimens. Although the use of type specimens for similar studies is extremely advantageous and should be encouraged, it must at the same time be tightly regulated because partial destruction of the specimens is required for DNA extraction. Molecular characters have added another dimension to taxonomic and phylogenetic investigations, and the utilization of herbarium, mummified and fossil specimens has greatly extended this effect.

PART III: FUTURE PLANS

We have completed the work on *Phialophora* (Yan et al., 1993a,b), but continue to investigate the intron that was found in this and other deuteromycetes (Rogers et al., 1993). We also continue work on molecular taxonomy and phylogenetics of several other deuteromycetes, which includes the use of herbarium type specimens. We have nearly completed the work on *Dermocybe*, relying exclusively on herbarium specimens. The work on *Cortinarius whiteae* is also nearing completion. The results will soon be published as three or four papers, possibly including a monograph for the genus *Dermocybe* in North America (to be authored by Dr. J.F. Ammirati).

Although data were not presented from our studies of mummified plant seeds, we continue to investigate various phenomena using seeds collected from packrat middens. The preservation of these seeds is often excellent. In the majority of cases we can extract DNA for PCR amplifications. Studies dealing with DNA condition, population structure, taxonomy, phylogenetics and genetic changes related to ecological changes have been initiated.

ACKNOWLEDGEMENTS

I would like to thank the following persons who contributed much of the work presented: For Part I: Arnie Bendich, Mary Jo Branthoover, Masaki Gondo, Mahmood Shivji and Geoff Spaulding; Part II: Joe Ammirati, Mari Shinohara, Yajuan Liu, C.J.K. (June) Wang and Zhong Hua Yan.

REFERENCES

Ammirati, J.F. 1972. The section *Dermocybe* of *Cortinarius* in North America. Ph.D. thesis, University of Michigan, Ann Arbor.

Ammirati, J.F. 1988. *Dermocybe* I: a preliminary treatment of species in the sections *Dermocybe, Holoxanthae* and *Malicoriae*. McIlvainea. (in press).

Ammirati, J.F. 1989a. *Dermocybe*, subgenus *Dermocybe*, section Sanguineae in Northern California. Mycotaxon 34:21-36.

Ammirati, J.F. 1989b. *Dermocybe* (Cortinariaceae, *Agaricales*): North American names - new combinations, synonyms and notes. Mycotaxon 33:473-446.

Ammirati, J.F and Gilliam, M.S. 1975. *Cortinarius*, section *Dermocybe*. Further studies on *Cortinarius aureifolius*. Sydowia Beiheft zur Nova Hedwigia 51:39-52.

Ammirati, J.F. and Smith, A.H. 1969. Studies in the genus *Cortinarius* I: section *Dermocybe, Cortinarius aureifolius* complex. Michigan Botanist 8:175-180.

Ammirati, J.F. and Smith, A.H. 1977. Studies in the genus *Cortinarius* III: section *Dermocybe*, new North American species. Mycotaxon 5:381-397.

Ammirati, J.F. and Smith, A.H. 1978. Studies in the genus *Cortinarius* IV: section *Dermocybe*, new North American species. Mycotaxon 7:265-265.

Ammirati J.F. and Smith, A.H. 1984. *Cortinarius* II. A preliminary treatment of species in the subgenus *Dermocybe*, section Sanguineae, in North America north of Mexico. McIlvainea 6:54-64.

Anderson, J.B. and Petsche, D.M. 1986. Restriction polymorphisms in nuclear DNAs of the North American biological species of *Armillaria*. MSA Newsletter 37:17.

Anderson, J.B. and Smith, M.L. 1989. Variation in ribosomal and mitochondrial DNAs in *Armillaria* species, *in:* "Proceedings of the Seventh International Conference on Root and Butt Rot," D.J. Morrison, ed., IUFRO Working Party S2.06.01.

Anderson, J.B., Bailey, S.S., and Pukkila, P.J. 1990. Variation in ribosomal DNA among biological species of *Armillaria*, a genus of root-infecting fungi. Evolution 43:1652-1662.

Anderson, J.B., Petsche, D.M. and Smith, M.L. 1987. Restriction fragment polymorphisms in biological species of *Armillaria mellea*. Mycologia 79:69-76.

Appels, R. and Dvorak, J.1982. Relative rates of divergence of species and gene sequences within the rDNA region of species in Triticeae: implications for the maintenance of homogeneity of a repeated gene family. Theor. Appl. Genet. 63:361-365.

Arnold, N., Besl, H., Bresinsky, A. and Kemmer, H. 1987. Notizen zur Chemotaxonomie der Gattung *Dermocybe* (Agaricales) und zu ihrem Vorkommen in Bayern. Z. Mykol. 53:187-194.

Baker, S.B., Rugh, C.L. and Kamalay, J.C. 1990. RNA and DNA isolation from recalcitrant plant tissues. BioTechniques 9:268.

Bendich, A.J. 1985. Plant mitochondrial DNA: unusual variation on a common theme, *in:* "Genetic Flux in Plants," B. Hohn and E.S. Dennis, eds., Springer-Verlag, Vienna.

Borsuk, P.A., Naliec, M.M., Sepien, P.P. and Bartnik, E. 1982. Organization of the ribosomal RNA gene cluster in *Aspergillus nidulans*. Gene 17:147-152.

Bruns, T.D., Fogel, R., White, T.J. and Palmer, J.D. 1989. Accelerated evolution of a false-truffle from a mushroom ancestor. Nature 339:140-142.

Bruns, T.D. and Palmer, J.D. 1989. Evolution of mushroom mitochondrial DNA: *Suillus* and related genera. J. Mol. Evol. 28:349-362.

Bruns, T. D., Palmer, J.D., Schumard, D.S., Grossman, L.I. and Hudspeth, M.E.S. 1988. Mitochondrial DNAs of *Suillus*: three fold size change in molecules that share a common gene order. Curr. Genet. 13:49-56.

Bruns, T.D., White, T.J. and Taylor, J.W. 1991. Fungal molecular systematics. Annual Rev. Ecol. Syst. 22:525-564.

Cassidy, J.R., Moore, D., Lu, B.C. and Pukkila, P.J. 1984. Unusual organization and lack of recombination in the ribosomal RNA genes of *Coprinus cinereus*. Curr. Genet. 8:607-613.

Conant, N.F. 1937. The occurrence of a human pathogenic fungus as a saprophyte in nature. Mycologia 29:597-598.

Davidson, R.W. 1935. Fungi-causing stain in logs and lumber in the Southern States, including five new species. J. Agric. Res. 50:789-807.

Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1:19.

Doyle, J.J. 1992. Gene trees and species trees: molecular systematics as one-character taxonomy. Syst. Bot. 17:144-163.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11.

Free, S.J., Rice, P.W. and Metzenberg, R.L. 1979. Arrangement of the genes coding for ribosomal ribonucleic acids in *Neurospora crassa*. J. Bacteriol. 137:1219-1226.

Gardes, M., Fortin, J.A., Mueller, G.M. and Kropp, R.B. 1990. Restriction fragment length polymorphisms in the nuclear ribosomal DNA of four *Laccaria* spp.: *L. bicolor, L. laccata, L. proxima* and *L. amethustina*. Phytopathology 80:1312-1317.

Harney, S.K., LoBuglio, K.F.,, Rogers, S.O. and Wang, C.J.K.1992. Identification of dematiaceous ecto- and ectendomycorrhizal fungi using RFLP analysis (Abstr.). Inoculum, Newslet. Mycol. Soc. Amer. 43:34.

Hawksworth, D.L., Sutton, B.C. and Ainsworth, G.C. 1983. "Ainsworth and Bisby's Dictionary of the Fungi. Ed. 7." Comm. Mycol. Inst., Kew. 445p.

Høiland, K. 1984. *Cortinarius*, subgenus *Dermocybe*. Opera Bot. 71:1-113.

Høiland, K. 1985. Contribution to the nomenclature of *Cortinarius* subgenus *Dermocybe*. Nord. J. Bot. 5:625-627.

Hoopes, B.C. and McClure, W.R. 1981. Studies on selectivity of DNA precipitation by spermine. Nucleic Acids Res. 9:5493.

Hughes, S.J. 1958. Revisiones hyphomycetum aliquot cum appendice de nominibus rejiciendis. Can. J. Bot. 36:727-836.

John, B. and Miklos, G. 1988. "The Eukaryote Genome in Development and Evolution," Allen and Unwin Publishers, Boston.

Kawasaki, M., Ishizaki, H., Nishimura, K. and Miyaji, M. 1990. Mitochondrial DNA analysis of *Exophiala jeanselmei* and *Exophiala dermatitidis*. Mycopathologia 110:107-112.

Keller, G. 1979. Chemotaxonomische pigmentations-untersuchungen in der Gattung *Dermocybe* (Fr.) Wünsche, Ph.D. thesis, Innsbruch, Austria.

Keller, G. 1982. Pigmentationsuntersuchungen bei europäischen Arten aus der Gattung *Dermocybe* (Fr.) Wünsche. Sydowia 53:110-126.

Kim, W.K., Mauthe, W., Hausner, G. and Klassen, G.R. 1990. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. Can. J. Bot. 68:1898.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X. and Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA, 86:6196.

Kohn, L.M. 1992. Developing new characters for fungal systematics: an experimental approach for determining the rank of resolution. Mycologia 84:139-153.

Lagerberg, T., Lundberg, G. and Melin, E. 1927. Biological practical researches in blueing in pine and spruce. Svenska Skogsvardsforen. Tidskr. 25:145-272.

Lewin, B. 1990. "Gene Expression, Vol. 2. Eucaryotic Chromosomes." John Wiley and Sons, New York.

Liu, Y.J., Ammirati, J.F. and Rogers, S.O. 1992. A comparison of relationships within the subgenus *Dermocybe* (Cortinariaceae) determined by molecular and morphological methods. (Abstr.). Inoculum, Newslet. Mycol. Soc. Amer. 43:41.

LoBuglio, K.F., Rogers, S.O. and Wang, C.J.K. 1991. Variation in ribosomal DNA among isolates of the mycorrhizal fungus *Cenococcum geophilum.* Can. J. Bot. 69:2331.

Lockington, R.A., Taylor, G.G., Winther, M., Scazzocchio, C. and Davies, R.W. 1982. A physical map of the ribosomal DNA repeat unit of *Aspergillus nidulans.* Gene 20:135-137.

Long, E.O. and Dawid, I.B. 1980. Repeated genes in eukaryotes. Ann. Rev. Biochem. 49:727-764.

Medlar, E.M. 1915. A new fungus, *Phialophora verrucosa*, pathogenic for man. Mycologia 7:200-203.

Melin, E. and Nannfeldt, J.A. 1934. Researches into the blueing of ground wood pulp. Svenska Skogsvardsforen. Tidskrift. 32:397-616.

Mitchell, T.G., White, T.J. and Taylor, J.W. 1992. Comparison of 5.8S ribosomal DNA sequences among the basidiomycetous yeast genera *Cystofilobasidium, Filbasidium* and *Filobasidiella.* J. Med. Veterinary Mycol. 30:207-218.

Möller, E.M., Bahnweg, G., Sandermann, H. and Geiger, H.H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20:6115.

Moser, M. 1955. Basidiomyceten II. Die Röhrlinge, Blätterpilze und Bauchpilze, *in:* "Gams Kleine Kryptogamenflora," Fischer, Stuttgart.

Moser, M. 1967. Basidiomyceten II. Die Röhrlinge und Blätterpilze, *in:* "Gams Kleine Kryptogamenflora," Fischer, Stuttgart.

Moser, M. 1972. Die Gattung *Dermocybe* (Fr.) Wünsche. Schweiz. Z. Pilzk. 50:153-167.

Moser, M. 1974a. Die Gattung *Dermocybe* (Fr.) Wünsche (Die Hautkopfe). Schweiz. J. Pilzk. 52:97-108.

Moser, M. 1974b. Die Gattung *Dermocybe* (Fr.) Wünsche (Die Hautkopfe), Schweiz. J. Pilzk. 52:129-142.

Moser, M. 1978. Basidiomycete II. Die Röhrlinge und Blätterpilze, *in:* "Gams Kleine Kryptogamenflora," Fischer, Stuttgart.

Moser, M. 1983. "Keys to Agarics and Boleti," R. Phillips (G. Fischer), Stuttgart, 535 pp.

Moser, M. 1986a. Cortinarius schaefferi Bresadola. Bol. Grup. Micol. G. Bresadola 29:100-104.

Moser, M. 1986b. *Dermocybe, in:* "The Agaricales in Modern Taxonomy," R. Singer, ed., Koeltz Scientific Books, Koenigstein, Germany.

Murray, H.G. and Thompson, W.F. 1980, Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8:4321.

Nelson, R.T., Yangeo, B.G., Strake, D.T. and Cochrane, B.J. 1990. Genetic studies in the genus *Basidiglobus*. II. Phylogenetic relationships inferred from ribosomal DNA analysis. Exper. Mycol. 14:197-206.

Orton, P.D. 1958. "*Cortinarius* II, The Naturalist (Mycological Section Yorkshire Naturalist's Union, Suppl. 2)."

Pallsboll, P.J., Vader, A., Bakke, I. and El-Gewely, M.R. 1992. Determination of gender in cetaceans by the polymerase chain reaction. Can. J. Zool. 70:2166.

Palmer, J.D. and Hebron, L.A. 1987. Unicellular structure of the *Brassica hirta* mitochondrial genome. Curr. Genet. 11:565-570.

Reeder, R.H. 1985. Mechanisms of nucleolar dominance in animals and plants. J. Cell Biol. 101:2013-2016.

Rehner, S.A. 1989. Biological speciation in the *Agrocybe pediades* species complex. Ph.D. thesis, University of Washington, Seattle, WA.

Rippon, J.W. 1988. "Medical Mycology. The Pathogenic Fungi and the Pathogenic Actinomycetes," W.B. Saunders Co., Philadelphia, 797p.

Robar, T.L., Harrison, K.A. and Grund, D.W. 1981. *Cortinarii* of Nova Scotia, I. Taxa in the subgenus *Dermocybe* (Fr.) Fr. Proc. N. S. Inst. Sci. 31:121-147.

Rogers, S.O. and Bendich, A.J. 1985. Extaction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Biol. 5:69.

Rogers, S.O. and Bendich, A.J. 1987. Ribosomal RNA genes in plants: variability in copy number and in the intergenic spacer. Plant Mol. Biol. 9:509-520.

Rogers, S.O. and Bendich, A.J. 1988. Extraction of DNA from plant tissues, *in:* "Plant Molecular Biology Manual," S. Gelvin and R.A. Schilperoort, eds., Kluwer Academic Publishers, Boston.

Rogers, S.O., Rehner, S.A., Bledsoe, C., Mueller, G.J. and Ammirati, J.F. 1989. Extraction of DNA from basidiomycetes for ribosomal DNA hybridizations. Can. J. Bot. 67:1235.

Rogers, S.O., Yan, Z.H., M.L. Shinohara, LoBuglio, K.F. and Wang, C.J.K. 1993. Messenger RNA intron in the 18s ribosomal RNA of deuteromycetes. Curr. Genet. 23:338-342.

Rohlf, F.J. 1992. "NTSYS-pc: Numerical taxonomy and multivariate analysis system, version 1.70 ". Exeter Publishing, Ltd., New York.

Russell, P.J., Wagner, S., Rodland, K.D., Feinbaum, R.L., Russell, J.P., Bret-Harte, M.S., Free, S.J. and Metzenberg, R.L. 1984. Organization of the ribosomal ribonucleic acid genes in various wild-type strains and wild-collected strains of *Neurospora*. Mol. Gen. Genet. 196:275-282.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Shinohara, M.L., LoBuglio, K.F., Wang, C.J.K. and Rogers, S.O. 1992. Characterization and evolutionary implications of an mRNA-like intron within the nuclear 18S ribosomal RNA gene of *Cenococcum geophilum* (Abstr.). Inoculum, Newslet. Mycol. Soc. Amer. 43.

Shumard, D.S., Grossman, L.I. and Hudspeth, M.E.S. 1986. Achlya mitochondrial DNA: gene location and analysis of inverted repeats. Mol. Gen. Genet. 202:16-23.

Shivji, M.S., Rogers, S.O. and Stanhope, M.J. 1992. Rapid isolation of high molecular weight DNA from marine macroalgae. Mar. Ecol. Prog. Ser. 84:197.

Specht, C.A., Novotny, C.P. and Ullrich, R.C. 1984. Strain specific differences in ribosomal DNA from the fungus *Schizophyllum* commune. Curr. Genet. 8:219-222.

Swofford, D. 1990. "PAUP: phylogenetic analysis using parsimony, Version 3.0." University of Illinois, Champaign.

Taylor, B. and Powell, A. 1982. Isolation of plant DNA and RNA. Focus 4:4.

Toothman, P., Rogers, S.O., Stuart, K. and Bendich, A.J. 1988. Species specificity of mitochondrial DNA flanking the 18S rRNA gene in two species of Cucurbitaceae. Plant Science 55:33-42.

Wang, C.J.K. and Zabel, R.A., eds., 1990. "Identification Manual for Fungi from Utility Poles in Northeastern United States." ATCC, Publisher, 356p.

Weitzman I., Gordon, M.A., Henderson, R.W. and Lapa, E.W. 1984. *Phialophora parasitica*, an emerging pathogen, *Sabouraudia.* J. Med. Veterinary Mycol. 22:331-339.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, *in:* "PCR Protocols: A Guide to Methods and Applications," M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, eds., Academic Press, Inc., New York.

Yan, Z.H., Rogers, S.O. and Wang, C.J.K. 1992. Relationship of species of *Phialophora* based on morphological and molecular characters (Abstr.). Inoculum, Newslet. Mycol. Soc. Amer. 43.

Yan, Z.H., Rogers, S.O., and Wang, C.J.K., 1993a. Molecular studies of Phialophora species, I: Restriction enzyme mapping of the 5.8S gene and internal transcribed spacers ribosomal DNA. Mycologia (in review).

Yan, Z.H., Rogers, S.O. and Wang, C.J.K. 1993b. Molecular studies of *Phialophora* species,II: Sequencing analysis of the 5.8S gene and internal transcribed spacers ribosomal DNA. Mycologia (in review).

Zolan, M.E. and Pukkila, P.J. 1986. Inheritance of DNA methylation in *Coprinus cinereus.* Mol. Cell Biol. 6:195.

Zweibel, S.M. and Wang, C.J.K. 1977. Re-examination of *Phialophora verrucosa, in:* "The black and white yeasts," Proceedings of the IV International Conference on the Mycoses, Brasilia, D.F., Brazil, 6-8 June.

DNA EXTRACTION FROM RECALCITRANT PLANTS: LONG, PURE, AND SIMPLE?

Kenneth J. Sytsma
Botany Department, University of Wisconsin
Madison, Wisconsin 53706, USA

SUMMARY

The extraction of DNA is reviewed in light of recalcitrant plant features, factors influencing choice of DNA extraction protocols, and solutions to problems in extracting DNA from recalcitrant plants. A flow chart is presented that summarizes major modifications of the CTAB method for use with recalcitrant species.

INTRODUCTION

Fourteen years ago Bendich and collaborators published one of the seminal papers on the extraction of DNA from plant tissue (Bendich et al., 1979). The title was provocative: "Plant DNA: Long, Pure, and Simple." A decade and a half and probably hundreds of thousands of plant DNA extractions later, it is clear that the extraction of pure and high molecular weight DNA is not always simple or routine. One only has to wait for the most recent issue of the *Plant Molecular Biology Reporter* to find the latest and most innovative protocol for the extraction of DNA from a specific group of recalcitrant plants (see reviews in Smith et al., 1991; Stein, 1993). Although some of these protocols are claimed to work efficiently across a broad diversity of plant taxa (Dellaporta et al., 1983; Doyle and Doyle, 1987, 1990; Guillemaut and Marechal-Drourard, 1992; Murray and Thompson, 1980), the increasing number of published protocols for specific troublesome groups (Gawal and Jarret, 1991; Honeycutt et al., 1992; Howland et al., 1991; Jhingan, 1992; Varadarajan and Prakash, 1991; Webb and Knapp, 1990) is ample evidence that a given method is not necessarily reproducible across groups and provides variable yields of DNA (Smith et al., 1991; Stein, 1993).

The isolation of pure and high molecular weight DNA is becoming a major concern on three counts. First, the need and demand for restriction fragment length (RFLP) analysis, construction of genomic libraries, amplification fragment length (AFLP or RAPD) analysis, DNA fingerprinting, and polymerase chain reaction (PCR) amplification of DNA for sequencing is accelerating at a phenomenal rate in the field of crop improvement (Burr et al., 1986; Doebley, 1992; Rieseberg and Brunsfeld, 1992). Second, molecular systematics and evolution, dependent on the extraction of DNA from a very wide diversity of plant taxa, is one of the fastest growing areas of research in the plant sciences (Clegg et al, 1992; Soltis et al., 1992a). This growth is exemplified by the phylogenetic analysis of epic proportions using chloroplast *rbc*L gene sequences of 499 species representing 265 families of flowering plants (Chase et al., 1993). Third, the escalating loss of the world's plant diversity has prompted the initiation of a "genetic insurance policy" in the form of an international genomic DNA-bank (Adams, 1990; Adams and Adams, 1993; Adams and Do, 1992). The development and utilization of simple and efficient DNA extraction methods for temperate and tropical species, wild and cultivated, and both tractable and recalcitrant, is now of the utmost importance and urgency.

RECALCITRANT PLANT FEATURES

Certain plant groups are notoriously difficult to extract DNA from their tissue (Baker et al., 1990; Couch and Fritz, 1990; Hughes and Galau, 1988; Smith et al., 1991; Stein, 1993; Webb and Knapp, 1990; Whitmore and Kriebel, 1987; personal observation; personal communication from many sources). These include, but are not restricted to, tropical members of the Magnoliales with high concentrations of phenolic secondary compounds, the Myrtales with various resins and mucilaginous compounds of unknown identity, a diverse assortment of families (e.g., Cactaceae, and members of the Malvales) with high levels of polysaccharides that co-precipitate with DNA, many, but not all, latex-bearing families, several of the monocot families with fibrous veined leaves, and even certain tropical cloud forest species with thick, tough, and leathery leaves (e.g., the coriaceous leaves of many Bombacaceae).

In surveying the literature dealing with DNA extraction techniques for this review, four types of compounds have been implicated or at least suspected as the prevailing cause for most problems in DNA extraction and subsequent DNA manipulation: proteins, polyphenolics, polysaccharides, and mucilages. These problems are aggravated by the use of older leaf tissue or tissue that has been dried or preserved (e.g., chemically dried, herbarium specimens, and fossils) (Chase and Hills, 1991; Liston et al., 1990; Pääbo, 1989; Rogers and Bendich, 1985; Rollo et al., 1987).

Inefficient lysis or high levels of nuclease activity can be problems in DNA extraction, but methods have been developed to counter them (Baker et al., 1990). More problematic are these aqueous-soluble byproducts of secondary metabolism, common especially in mature tissues, that interfere with the isolation of DNA when co-extracted during extraction. In living cells these compounds are compartmentalized in the organelles, vacuoles, and the cytoplasmic matrix. During lysis of either whole cells or separated organelles in DNA extraction, the compounds are released and come in contact with the DNA. The presence of free polyphenoloxidases can quickly brown and degrade DNA unless steps are taken to quickly remove, dilute, or bind these enzymes (Stein, 1993). Other proteins when oxidized can bind almost permanently to the exposed DNA, rendering later manipulations of the DNA difficult.

Polysaccharides are probably the most common reason for low yields and purity of DNA (Do and Adams, 1991; Fang et al., 1992). About 90% of the cell wall is composed of cellulose, pectins, and related polysaccharides and it is these compounds that render the extraction of DNA from plant tissue so different (and more difficult) than from animal tissue (Jhingan, 1992). Restriction digestion and PCR of DNA are especially sensitive to the presence of acidic polysaccharides (Demeke and Adams, 1992; Do and Adams, 1991). Mucilaginous compounds, often of unknown constituents but perhaps related to polysaccharides, are especially problematic in certain families by preventing efficient homogenization of plant cells, by binding with DNA and causing DNA loss, and hindering resuspension of DNA.

FACTORS INFLUENCING CHOICE OF DNA EXTRACTION PROTOCOL

To overcome these various problems in recalcitrant plants, one must initially consider several factors that may well have an impact on what DNA extraction strategies are employed. First, the choice of plant tissue is extremely important. Although leaf or frond tissue is the most common source of DNA, alternative sources are sometimes necessary and often exhibit different yields and purity of DNA. Stem and seed tissue have been used in instances where leaf tissue is not possible to obtain (e.g., *Koeberlinia*

[R. Price, personal communication] and *Clarkia* [unpublished data], respectively). In the latter, seed tissue was considerably easier than leaf tissue as a source for DNA. Herbarium and fossil sources are also increasingly be used as sources for DNA, present unique difficulties (therefore recalcitrant), and thus require different approaches to solving DNA extraction problems (Golenberg et al., 1990; Pääbo, 1989; Pääbo and Wilson, 1991; Rogers and Bendich, 1985; Rollo et al., 1987; Soltis et al. 1992b; see Golenberg, this volume; see Rogers, this volume).

Secondly, preservation of material whether frozen, lyophilized, silica gel dried, or NaCl-CTAB saturated can have a dramatic impact on the yield and purity of extracted DNA, and limit what molecular tools can be used later (Adams and Do, 1992; Chase and Hills, 1991; Liston et al., 1990; Pyle and Adams, 1989; Rogstad, 1992; Tai and Tanksley, 1990). The choice of preservation can not only require modifications of the DNA extraction method, but also can eliminate certain methods as even feasible. For example, silica-gel dried leaf tissue requires the use of lower quantities of starting tissue in CTAB "minipreps" (less than 0.3g often; M. Chase, personal communication), and prevents the use of "macropreps" (e.g., Zimmer et al., 1981; unpublished data). In general, an experimental and comparative study of the effects of these various preservation methods on subsequent DNA extraction and manipulation is sorely needed. Specifically, as regards to recalcitrant plant species, the collection and utilization of fresh and actively growing leaf tissue when and wherever possible is recommended to overcome many of these preservation effects (Sytsma et al., 1993).

Thirdly, the need for either specific organellar DNAs or total cellular DNA must be considered. Although methods do exist for the separate isolation of nuclei, chloroplasts, and mitochondria (Milligan, 1989; Palmer and Stein, 1982; Stein, 1993), the isolation of purified organellar DNA is generally far more difficult in recalcitrant plants than is the isolation of total cellular DNA. Moreover, the latter provides a complete genetic bank and permits subsequent analysis of any organellar DNA via Southern hybridization, cloning, or PCR.

Fourthly, the specific DNA technology to be used should influence the choice of tissue preservation and DNA extraction. Restriction endonuclease utilization (Dowling et al., 1990; Doyle and Dickson, 1987) requires fairly clean and, in most cases, large quantities of DNA. Random amplified polymorphic DNA and related techniques (Welsh and McClelland, 1990; Williams et al., 1990) require less amounts of DNA, but purity is necessary to ensure repeatability and confidence in cross DNA comparison. PCR amplification of specific targeted DNAs (Erlich, 1989; Mullis et al., 1986) requires very little template DNA. PCR is certainly affected by specific contaminants (Demeke and Adams, 1992), but the repeated successful PCR amplification of DNA from degraded fossil, herbarium, and recalcitrant plant species (Chase et al., 1993; Golenberg et al., 1990; Rodman et al., 1993) is confirmation of the wide applicability of the technique. Indeed, a number of rapid "microprep" DNA extraction methods for very small amounts of tissue have been developed and used successfully for PCR amplification (Berthomieu and Meyer, 1991; Edwards et al., 1991; Langridge et al., 1991; Luo et al., 1992).

SOLUTIONS TO PROBLEMS IN EXTRACTING DNA FROM RECALCITRANT PLANTS

Few comparative studies have been done that examine the relative merits of different DNA extraction methods for a number of different recalcitrant plant species (Smith et al., 1991). Two major types of extraction protocols are routinely used: (1)

"macropreps" requiring 10-100g of leaf tissue, homogenization of tissue and separation of organelles, lysis of organelles, and finally precipitation of DNA (e.g., Zimmer et al., 1981); and (2) "minipreps" requiring 2g or less of tissue, immediate and simultaneous lysis of tissue, cells, and organelles, separation of aqueous-nucleic acid phase, and finally precipitation of DNA (e.g., Dellaporta et al., 1983; Doyle and Doyle, 1987; Murray and Thompson, 1980; Rogers and Bendich, 1985; Saghai-Maroof et al., 1984). The advantages of the "macroprep" include separation of organelles from secondary compounds from the cytosol or vacuole prior to lysis, but a disadvantage is the relatively lower yields and necessity for larger amounts of starting tissue. The advantages of "miniprep" include efficiency, yield, and the small amount of required tissue, but a disadvantage is the immediate exposure of DNA to secondary compounds, proteins, and nucleases. These two major types of extraction protocols are not always that different. For example, both can utilize CsCl gradients to purify the DNA from RNA and secondary compounds/proteins, and "hybrid" protocols have been developed that utilize advantages of both methods (e.g., Smith et al., 1991).

The preferred method of DNA extraction for a given recalcitrant plant can only be determined through trial and error. In a recent comparative study of six DNA extraction methods (a "macroprep", several "minipreps", and a hybrid prep) on liquid N_2-powdered, fresh tissue of eight species, including several considered somewhat or severely recalcitrant, no one method proved superior (Smith et al., 1991). However, these results (as well as results communicated to the author by a good number of researchers) suggest that the CsCl "macroprep" method of Zimmer et al. (1981), with modifications suggested by Smith et al. (1991), and the 2% CTAB "miniprep" method of Doyle and Doyle (1987) are the two most logical starting points when searching for DNA extraction methods that will work for a particular recalcitrant species. Because of the efficiency and relatively low amounts of leaf tissue needed in the CTAB "minipreps", the latter method is recommended for the initial screening and the discussion below will be based on modifications of the CTAB methods (i.e., Doyle and Doyle, 1987) to circumvent specific problems encountered with recalcitrant plants (Table 1). Alternative, and perhaps new, methods for further screening will be presented and discussed at the end.

Yield Problems

The amount of DNA recovered from CTAB extraction procedures (like any method) varies tremendously (personal observations). However, yield is not critical for extraction of 10s if not 100s of micrograms of DNA. Yield can be enhanced in many cases by using the freshest and youngest leaf tissue possible. Reducing the amount of tissue per volume of CTAB extraction buffer increases the relative yield, and in fact can often increase the total yield of DNA. In the experience of my laboratory, over zealous use of tissue to maximize DNA yields can often be counter-productive and causes the precipitation of large quantities of polysaccharides making DNA resuspension difficult. For certain recalcitrant genera in the Onagraceae (e.g., *Clarkia*), we have found that seed tissue increases both the yield and purity of DNA relative to even young leaf tissue. The ratio of chloroplast DNA to nuclear DNA in these extractions is significantly lowered, however. Similarly, Baker et al. (1990) showed that the source of tissue was the primary factor in determining yield of both DNA and RNA. In their survey of both angiosperm PCR and related manipulations, restriction site analysis is dependent on the successful (*Gossypium*) and gymnosperm (*Pinus*) recalcitrant species.

Table 1. Survey of modifications to the basic 2% CTAB DNA extraction procedures of Murray and Thompson (1980), Saghai-Maroof et al. (1984) and Doyle and Doyle (1987) for problems with DNA yield and purity in recalcitrant plants. Numbers refer to literature citations at bottom of the table.

	Yield	Purity				
		General/ Proteins	Polyphenolics	Mucilages	Polysaccharides	Enzymatic Activity
Tissue preparation						
Different tissues	1,15,!	!	!	!	!	!
Polytron homogenization	23, !					
Increase buffer:tissue	2,*,!	2,20,*,!		!		
Organelle preseparation			20,!	20,!	20,!	20,!
Extraction buffer						
Lower % CTAB	2,11,19,23			2		
Higher % CTAB	9,11,20,!			9,20,!		
High ionic strength	15,18					
PVP-40 or PVPP			2,3,12,14,19,!		!	
PEG		13				
high pH (8.5)	15					
low pH (5.5)			12			
High [β-mercap]	11,20,22,!	11,20,22,!		20,22,!		
Dithiothreitol	15					
Diethyldithio carbamic acid			3,14			
Na-bisulfite		21,**				
cysteine			12			
Spermidine		13				
Organic/aqueous separation						
Miracloth pre-separation	1,11	1,11				
Phenol wash		2,23	22			
No phenol wash	1,15					
Long chloroform wash (30min)	2,*					
Repeated chloroform steps		10,13,14,22,*				
CsCl ultracentrifugation	1	1,14	!	!	!	
DNA precipitation						
Ethanol (not isopropanol)	2,!	2,10,!				
NH_4OAc (not NaOAc)		2,20,!				
Repeated precipitations		15,20,22,!			15	
2+M NaCl (or KCl)		11,17,!			10	10,17
Overnight precipitation	2					
DNA purification						
Resin columns/tips		12			7,8	7,8,12
Gel elution						2
Proteinases		!				
Alternative extraction protocols						
8M Urea	22		22			
SDS	1,6,12,15,21,22					
Organic solvents	4,5	4,5	4,5	4,5	4,5	4,5
Xanthates	16	16	16	16	16	16

1:Baker et al., 1990; 2:Bult et al., 1992; 3:Couch and Fritz, 1990; 4:Dally and Second, 1989; 5:deKocho and Hamon, 1990; 6:Dellaporta et al., 1983; 7:Demeke and Adams, 1992; 8:Do and Adams, 1991; 9:Doyle and Doyle, 1990; 10:Fang et al., 1992; 11:Gawal and Jarret, 1991; 12:Guillemaut and

Marechal-Drouard, 1992; 13:Honeycutt et al., 1992; 14:Howland et al., 1991; 15:Hughes and Galau, 1988; 16:Jhingan, 1992; 17:Luo et al., 1992; 18:Perez et al., 1990; 19:Rogers and Bendich, 1985; 20:Smith et al., 1991; 21:Tai and Tanksley, 1990; 22:Varadarajan and Prakash, 1991; 23:Webb and Knapp, 1990; *:M. Chase, pers. comm.; **:M. Murray, pers. comm.; !:unpublished data.

With many monocots, we have found that increasing CTAB to 6% and NaCl to 2.1M dramatically increases the yield of total cellular DNA (see Smith et al., 1991 for protocol). Conversely, Bult et al. (1992) claim that reducing CTAB to 1% increases the lytic ability of the extraction buffer when dealing with mucilaginous species. The use of polytron homogenization of nitrogen powdered tissue in CTAB buffer prior to the high temperature incubation has shown a 4-6 fold increase in yield for some recalcitrant plants (Webb and Knapp, 1990). The substitution of EtOH for isopropanol in the DNA precipitation step has been shown to increase yield substantially in many instances (Bult et al., 1992; Rogers and Bendich, 1985; personal observations). Other methods to increase the yield during DNA precipitation have been suggested (see Table 1; Doyle and Doyle, 1987; E. Golenberg, S. Rogers, personal communications): increasing the length of time at -20°C or colder, adding carriers such as tRNAs or glycogen, and using spermine for more efficient DNA precipitation.

If these alterations do not significantly improve DNA yield, other DNA extraction methods should be pursued. "Macropreps" requiring 10g or greater amounts of leaf tissue are often very effective (Smith et al., 1991; Zimmer et al., 1981). This protocol requires effective homogenization (using a polytron), separation of organelles from the tissue and cellular matrix, lysis of organelles, and subsequent CsCl-EtBr banding via ultracentrifugation. Speed and efficiency are sacrificed, but total yield (not relative yield) and purity are usually increased significantly. Other baseline "miniprep" methods (e.g., Dellaporta et al., 1983) and their spinoffs should also be tested. Alternatively, a hybrid method involving the organellar separation of "macroprep" methods and the small scale and efficient steps of the CTAB method could be investigated (Smith et al., 1991). This method was requisite for the successful extraction of DNA from mature leaves of many Hawaiian Lobeliaceae (Givnish et al., 1993), plants with high concentrations of both mucilages and latex.

Purity Problems

No matter what the reasons for the problems in DNA purity, three minor changes to many published CTAB extraction procedures can often increase purity of the DNA (Table 1). First, ethanol rather than isopropanol in the precipitation step not only enhances yield but also purity (Rogers and Bendich, 1985; Bult et al., 1992). Second, the use of ammonium acetate instead of, or as a prior step to, sodium acetate in the DNA precipitation step has been shown to increase the purity of the final resuspended DNA (see *Focus* (BRL) 4 (3):12). DNA is precipitated in the presence of 2.5M NH_4OAc and EtOH (i.e., the volume of the aqueous DNA solution is measured, 1/2 volume of 7.5M NH_4OAc is added, and 2-3 volumes (final) of EtOH). Third, increasing the number of DNA precipitation steps and resuspensions (up to three) can effectively purify many DNA samples (personal observations and communications). Additional, and usually more elaborate and time-consuming, changes to the basic 2% CTAB procedure (Doyle and Doyle, 1987; Saghai-Maroof et al., 1984) or to SDS detergent procedures (Dellaporta et al., 1983) can be effective, but these are often geared to specific problems in DNA extraction and will be dealt with individually below (see Table 1 for a more extensive listing of solutions to many of these problems). The review of Stein (1993) is

particularly recommended for obtaining information on modifications of extraction procedures in specific plant groups and on the advantages and disadvantages of these modifications.

Proteins. Careful chloroform washes and separation of the aqueous and organic phases are often sufficient for eliminating problems with proteins. Dramatic increases in both yield and purity have been noted with simply allowing the chloroform wash to take place on a gentle shaker for up to 30 minutes (M. Chase, personal communication). In some recalcitrant plants, however, phenol and/or phenol-chloroform washes are necessary, these often most effective if done early in the DNA extraction protocol (Bult et al., 1992). Proteinases are occasionally used either during lysis or DNA purification steps (Stein, 1993). As a last resort, CsCl ultracentrifugation might be necessary (Baker et al., 1990; Howland et al., 1991).

Polyphenols. Recalcitrant plants or mature or old leaf tissue often exhibit high levels of tannins and other polyphenolic secondary compounds that become oxidized and damage DNA. The addition of various antioxidants other than B-mercaptoenthanol such as dithiothreitol, sodium bisulfite, or diethyldithiocarbamic acid (DEDTCA) to extraction buffers have made possible the removal of DNA from such recalcitrant plant tissue as mature *Betula* leaves (Howland et al., 1991). DEDTCA acts simply as a competitor against oxygen for the copper center of polyphenoloxidases, thus preventing oxidation of the polyphenols and subsequent damage to attached DNA (Howland et al., 1991). Decreasing the pH of the extraction buffer to 5.5, as a means of reducing ionization, has been shown to be effective (Guillemaut and Marechal-Drouard, 1992). The addition of polyvinylpyrrolidone (PVP) or polyvinylpolypyrrolidone (PVPP), acting as chelators for various secondary compounds, is now routinely used in the extraction of DNA from recalcitrant plants (Table 1).

Mucilages. The most straightforward, although longer, method to eliminate mucilaginous compounds during DNA extraction is to separate organelles from cellular contents prior to lysis (Smith et al., 1991). This method has dramatically increased both the yield and purity of DNAs extracted from Lobeliaceae and Onagraceae (Givnish et al., 1993; unpublished data). Increasing the amount (sometimes up to 200ul per 10ml extraction buffer, i.e., 2%) of β-mercaptoenthanol in the normal CTAB procedure is effective in reducing the amount of mucilage encountered during later steps in the extraction process (Smith et al., 1991; Varadarajan and Prakash, 1991). However, although the increased β-mercaptoenthanol proved effective for *Ipomoea* (Gawal and Jarret, 1991; Varadarajan and Prakash, 1991), concentrations greater than 0.1% reduced yields in *Musa* (Gawal and Jarret, 1991). Similarly, both lower (0.5-1.0%) and higher CTAB concentrations (3-6%) have been suggested as useful for reducing levels of mucilages in families of the Myrtales (Bult et al., 1992; Doyle and Doyle, 1990; Smith et al., 1991; unpublished data). The considerably higher molarity of NaCl (2.1 vs. 1.4M) in the 3-6% CTAB extraction buffers (Smith et al., 1991) might actually be the causative agent for the reduction seen in mucilage (see below), and the role of high salt in reducing mucilage as well as polysaccharides needs to be clarified. Organic solvents have been used with some success to bypass the sticky mucilaginous component in some recalcitrant plants (most notably Cactaceae) (Stein, 1993; R. Wallace, personal communication). These solvents, trichlorobenzene and decahydronaphthalene, were used in a modified procedure based on Dally and Second (1989).

Polysaccharides. Cellular wall celluloses and pectins represent the class of polysaccharides most likely to co-precipitate with DNA and causing problems both in

yield and purity (Demeke and Adams, 1992; Do and Adams, 1991; Fang et al., 1992; Jhingan, 1992). Separation of organelles prior to lysis markedly reduces these problems (Smith et al., 1991). Increasing salt concentrations in either the DNA extraction buffer (see mucilages above) or DNA precipitation steps (Fang et al., 1992) removes much of the contaminating polysaccharides. Concentration of NaCl (and possibly KCl) greater than 1.5M remove polysaccharides and permit subsequent enzymatic manipulation of the DNA (Fang et al., 1992). Not surprisingly, resin-tip elution procedures have been shown to be quite effective in removing contaminating polysaccharides, due in part most likely to the high NaCl wash steps (Demeke and Do, 1992; Do and Adams, 1991).

DISCUSSION

It should be obvious that DNA extraction from recalcitrant plants is not simple nor does it always provide long and pure DNA. But, it is also true that a substantial number of modifications and new protocols have and are being developed for particularly recalcitrant plant species (Table 1). The effectiveness of these modifications and alternative protocols needs further experimental work. Thus, the blanks in Table 1 do not necessarily indicate negative results of particular modifications on either yield or purity, and some of these blanks might well be filled in with future or presently unpublished results. A flow chart for the sometimes tedious task of modifying the basic CTAB extraction method is provided in Figure 1. Although the CTAB procedure in its multitude of variations (Doyle and Doyle, 1987; Murray and Thompson, 1980; Rogers and Bendich, 1985; Saghai-Maroof et al., 1984) is recommended as a starting point in dealing with recalcitrant plant species, other established as well as new methods are certainly worth exploring if these CTAB -based suggestions do not work.

Two suggestions for DNA collection for the DNA-Bank program can be offered. First, tissue preservation *along* with DNA extraction has to be considered in collection programs. The method of tissue preservation is probably the most critical factor with regards to the extraction of high molecular weight and purified DNA. Second, "black box" DNA extractions *en masse* is not yet feasible or desired. The wide varieties of modifications necessary for specific recalcitrant plant groups is ample proof that no one method has been identified that will work successfully and routinely across most plant groups. Time and efficiency must be sacrificed, at least presently, for the sake of obtaining sufficient quantities of quality DNA from the world's plant biodiversity.

ACKNOWLEDGEMENTS

This survey and both the published and unpublished DNA extraction tests were supported by NSF grants BSR-8806520, 8815173, 9007293, 9020055, and 9016260, by a National Geographic Society research grant, and by support from the Nave Foundation, University of Wisconsin. The unpublished results and suggestions provided by M. Chase, M. Murray, S. Rogers, D. Stein, R. Wallace and various participants of the 2nd International DNA Bank-Net Meeting are gratefully acknowledged. The repeated DNA extraction tests done and results provided by D. Baum, E. Conti, T. Evans, T. Givnish, S. Graham, W. Hahn, K. Karol, M. Kuckenreuther, M. Nepokroeff, J. Rodman, J. Smith, and R. Smith make up many of the unpublished modifications presented here.

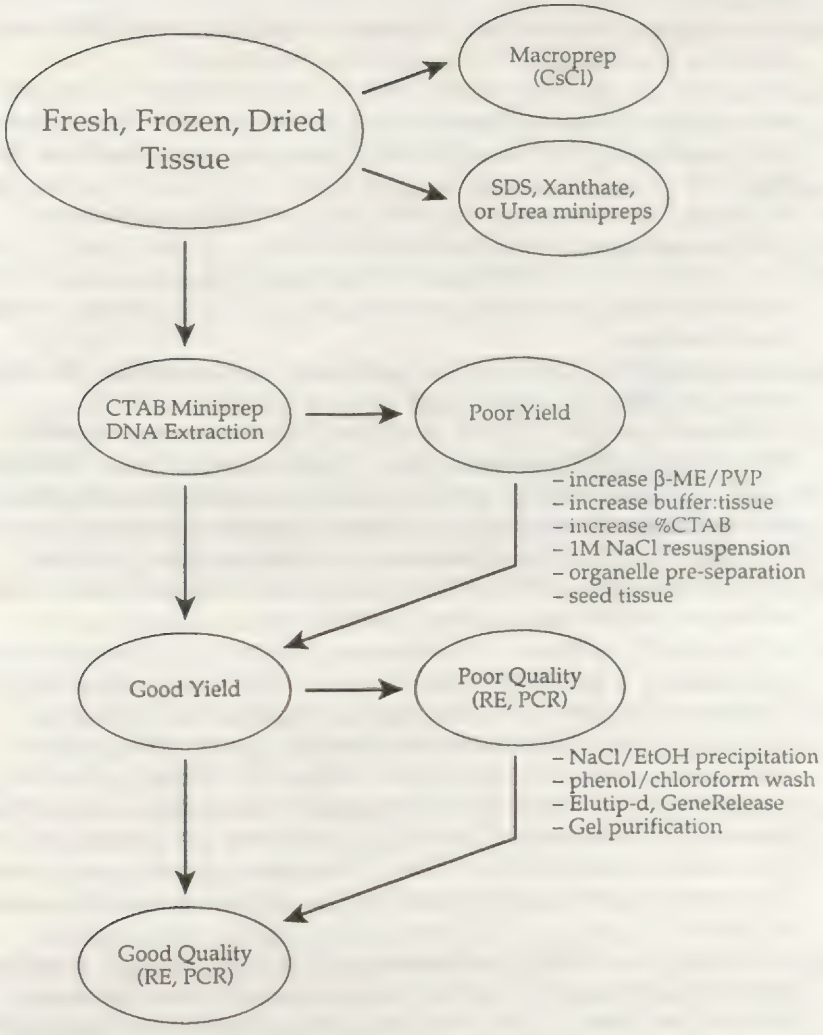


Figure 1. Flow chart summarizing major modifications of the CTAB "miniprep" procedures of Saghai-Maroof et al. (1984) and Doyle and Doyle (1987) for use with recalcitrant plant species. Decisions that impact on the flow chart include tissue type, preservation of tissue, and molecular techniques to be used.

REFERENCES

Adams, R.P. 1990. The preservation of Chihuahuan plant genomes through *in vitro* biotechnology: DNA-Bank Net, a genetic insurance policy, *in:* "Third Symposium on Resources of the Chihuahuan Desert Region," A.M. Powell, R.R. Hollander, J.C. Barlow, W.B. McGillivray and D.J. Schmidly, eds., Printech Press, Lubbock, TX.

Adams, R.P. and Adams, J.E. Eds. 1993. "Conservation of Plant Genes: DNA Banking and *in vitro* Biotechnology." Academic Press, San Diego, CA.

Adams, R.P. and Do, N. 1992. Preservation of DNA in plant specimens from tropical species by desiccation, *in:* "Conservation of Plant Genes: DNA banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, New York.

Baker, S.S., Rugh, C.L. and Kamalay, J.C. 1990. RNA and DNA isolation from recalcitrant plant tissues. BioTechniques 9:268-272.

Bendich, A.J., Anderson, R.S. and Ward, L.B. 1979. Plant DNA: long, pure and simple, *in:* "Genome Organization and Expression in Plants," C.J. Leaver, ed., Plenum Press, New York.

Berthomieu, P. and Meyer, C. 1991. Direct amplification of plant genomic DNA from leaf and root pieces using PCR. Plant Molec. Biol. Reptr. 17:55-557.

Bult, C., Kallersjo, M. and Suh, Y. 1992. Amplification and sequencing of 16S/18S rDNA from gel-purified total plant DNA. Plant Molec. Biol. Reptr. 10:272-284.

Burr, B., Evola, S.V. and Burr, F.A. 1986. The applications of restriction fragment polymorphisms to plant breeding, *in:* "Genetic Engineering: Principles and Methods," J.K. Setlow and A. Hollaender, eds., Plenum Press, New York.

Chase, M.W. and Hills, H.H. 1991. Silica gel: an ideal material for field preservation of leaf samples for DNA studies. Taxon 40:215-220.

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan, D., Les, D.H., Mishler, B.D., Duvall, M.R., Price, R.A., Hills, H.G., Qiu, Y., Kron, K.A., Rettig, J.H., Conti, E., Palmer, J.D., Manhart, J.R., Sytsma, K.J., Michaels, H.J., Kress, W.J., Karol, K.G., Clark, W.D., Hedrén, M., Gaut, B.S., Jansen, R.K., Kim, K.-J., Wimpee, C.F., Smith, J.F., Furnier, G.R., Strauss, S.H., Xiang, Q., Plunkett, G.M., Soltis, P.S., Swensen, S.M., Williams, S.E., Gadek, P.A., Quinn, C.J., Eguiarte, L.E., Golenberg, E., Learn, G.H.,Jr., Graham, S.W., Barrett, S.C.H., Dayanandan, S. and Albert, V.A. 1993. Phylogenetics of seed plants: An analysis of nucleotide sequences from the plastid gene *rbc*L. Ann. Missouri Bot. Gard. 80:528-580.

Clegg, M.T., Felsenstein, J., Fitch, W., Goodman, M., Hillis, D., Rilley, M., Ruddle, F., Sankoff, D., Azberger, P., Courtnsey, M., Harriman, P., Lynch, C., Plesset, J., Weiss, M. and Yates, T. 1992. Position paper on molecular evolution. Molec. Phylog. Evol. 1:84-85.

Couch, J.A. and Fritz, P.J. 1990. Isolation of DNA from plants high in phenolics. Plant Molec. Biol. Reptr. 8:8-12.

Dally, A.M. and Second, G. 1989. Chloroplast DNA isolation from higher plants: an improved non-aqueous method. Plant Molec. Biol. Reptr. 7:135-43.

deKocho, A. and Hamon, S. 1990. A rapid and efficient method for the isolation of total DNA from plants of the genus *Abelomoschus.* Plant Molec. Biol. Reptr. 8:3-7.

Dellaporta, S.L., Wood, J. and Hicks, J.B. 1983. A plant DNA minipreparation: Version II. Plant Molec. Biol. Reptr. 1:19-21.

Demeke, T. and Adams, R.P. 1992. The effects of plant polysaccharides and buffer additives on PCR. BioTechniques 12:332-334.

Do, N. and Adams, R.P. 1991. A simple technique for removing plant polysaccharide contaminants from plant DNA. BioTechniques 10:162-166.

Doebley, J. 1992. Molecular systematics and crop evolution, *in:* "Molecular Systematics of Plants," P.S. Soltis, D.E. Soltis and J.J. Doyle, eds, Chapman & Hall, New York.

Dowling, T.E., Moritz, C. and Palmer, J.D. 1990. Nucleic acids II: restriction site analysis, *in:* "Molecular Systematics," D.M. Hillis and C. Moritz, eds., Sinauer, Sunderland, MA.

Doyle, J.J. and Dickson, E.E. 1987. Preservation of plant samples for DNA restriction endonuclease analysis. Taxon 36:715-722.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. 19:11-15.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Edwards, K., Johstone, C. and Thomson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.

Erlich, H.A. 1989. "PCR Technology: Principles and Applications for DNA Amplification." Stockton Press, New York.

Fang, G., Hammar, S. and Grumet, R. 1992. A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques 13:52-56.

Gawal, N.J. and Jarret, R.L. 1991. A modified CTAB DNA extraction procedure for *Musa* and *Ipomoea.* Plant Molec. Biol. Reptr. 9: 262-266.

Givnish, T.J., Sytsma, K.J., Smith, J.F. and Hahn, W.J. 1993. Molecular evolution, adaptive radiation and geographic speciation in *Cyanea* (Campanulaceae), a species-rich genus of Hawaiian plants, *in*: "Origin and Adaptive Radiation of the Hawaiian Biota," W.L. Wagner and V.L. Funk, eds., Smithsonian Institution, Washington, D.C.

Golenberg, E.M., Giannasi, D.E., Clegg, M.T., Smiley, C.J., Durbin, M., Henderson, D. and Zurawski, G. 1990. Chloroplast DNA sequence from a Miocene *Magnolia* species. Nature 344:656-658.

Guillemaut, P. and Marechal-Drouard, L. 1992. Isolation of plant DNA: a fast, inexpensive, and reliable method. Plant Molec. Biol. Reptr. 10:60-65.

Honeycutt, R.J., Sobral, B.W.S., Keim, P. and Irvine, J.E. 1992. A rapid DNA extraction method for sugarcane and its relatives. Plant Molec. Biol. Reptr. 10:66-72.

Howland, D.E., Oliver, R.P. and Davy, A.J. 1991. A method of extraction of DNA from birch. Plant Molec. Biol. Reptr. 9:340-344.

Hughes, D.W. and Galau, G. 1988. Preparation of RNA from cotton leaves and pollen. Plant Molec. Biol. Reptr. 6:253-257.

Jhingan, A.K. 1992. A novel technology for DNA isolation. Meth. Mol. Cell. Biol. 3:15-22.

Langridge, U., Schwall, M. and Langridge, P. 1991. Squashes of plant tissue as substrate for PCR. Nucleic Acids Res. 19:6954.

Liston, A., Rieseberg, L.H., Adams, R.P., Do, N. and Zhu, G-L. 1990. A method for collecting dried plant specimens for DNA and isozyme analyses, and the results of a field test in Xinjiang, China. Ann. Missouri Bot. Gard. 77:859-863.

Luo, G., Hepburn, A.G. and Widholm, J.M. 1992. Preparation of plant DNA for PCR analysis: a fast, general and reliable procedure. Plant Molec. Biol. Reptr. 10:319-323.

Milligan, B.G. 1989. Purification of chloroplast DNA using hexadecyltrimethyl-ammonium bromide. Plant Molec. Biol. Reptr. 7:144-149.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G. and Erlich, H. 1986. Specific enzymatic amplification of DNA *in vitro*: the polymerase chain reaction. Cold Spring Harbor Symp. Quant. Biol. 51:263-273.

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight DNA. Nucleic Acids Res. 8:4321-4325.

Pääbo, S. 1989. Ancient DNA: extraction, characterization, molecular cloning and enzymatic digestion. Proc. Natl. Acad. Sci. USA 86:1939-1943.

Pääbo, S. and Wilson, A.C. 1991. Miocene DNA sequences--A dream come true? Curr. Biol. 1:45-46.

Palmer, J.D. and Stein, D.B. 1982. Chloroplast DNA from the fern *Osmunda cinnamomea:* physical organization, gene localization and comparison to Angiosperm chloroplast DNA. Curr. Genet. 5:165-170.

Perez, C., Bonavent, J.F. and Berville, A. 1990. Separation of mitochondrial DNAs from sunflowers (*Helianthus annuus* L.) and from beets (*Beta vulgaris*) using a medium with a high ionic strength. Plant Molec. Biol. Reptr. 8:104-113.

Pyle, M.M. and Adams, R.P. 1989. *In situ* preservation of DNA in plant specimens. Taxon 38:576-581.

Rieseberg, L.H. and Brunsfeld, S.J. 1992. Molecular evidence and plant introgression, *in:* "Molecular Systematics of Plants," P.S. Soltis, D.E. Soltis and J.J. Doyle, eds., Chapman & Hall, New York.

Rodman, J., Price, R., Karol, K., Conti, E., Sytsma, K.J. and Palmer, J.D. 1993. Gene sequence evidence for monophyly of mustard oil families. Ann. Missouri Bot. Gard. (in press).

Rogers, S.O. and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Molec. Biol. Reptr. 5:69-76.

Rogstad, S.H. 1992. Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analyses. Taxon 41:701-708.

Rollo, F., La Marca, A. and Amici, A. 1987. Nucleic acids in mummified plant seeds: screening of twelve specimens by gel-electrophoresis, molecular hybridization and DNA cloning. Theor. Appl. Genet. 73:501-505.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W.. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Smith, J.F., Sytsma, K.J., Shoemaker, J.S. and Smith, R.L. 1991. A qualitative comparison of total cellular DNA extraction protocols. Phytochem. Bull. 23:2-9.

Soltis, P.S., Soltis, D.E. and Doyle, J.J. Eds. 1992a. "Molecular Systematics of Plants," Chapman and Hall, New York.

Soltis, P.S., Soltis, D.E. and Smiley, C.J. 1992b. An *rbc*L sequence from a Miocene *Taxodium* (Bald Cypress). Proc. Natl. Acad. Sci. USA 89:449-451.

Stein, D.B. 1993. Isolating and comparing nucleic acids from land plants: nuclear and other organellar genes, *in:* "Molecular Evolution: Producing the Biochemical Data," E.A. Zimmer, T.J. White, R.L. Cann and A.C. Wilson, eds., Meth. Enzymol. 224 153-167.

Sytsma, K.J., Givnish, T.J., Smith, J.F. and Hahn, W.J. 1993. Obtaining and storing land plant samples for macromolecular comparisons, *in:* "Molecular Evolution: Producing the Biochemical Data," E.A. Zimmer, T.J. White, R.L. Cann and A.C. Wilson, eds., Meth. Enzymol. 224:23-37.

Tai, T.H. and Tanksley, S.D. 1990. A rapid and inexpensive method for isolation of total DNA from dehydrated plant tissue. Plant Molec. Biol. Reptr. 8:297-303.

Varadarajan, G.S. and Prakash, C.S. 1991. A rapid and efficient method for the extraction of total DNA from the sweet potato and its related species. Plant Molec. Biol. Reptr. 9:6-12.

Webb, D.M and Knapp, S.J. 1990. DNA extraction from a previously recalcitrant plant genus. Plant Molec. Biol. Reptr. 8:180-185.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.

Whitmore, F.W. and Kriebel, H.B. 1987. Expression of a gene in *Pinus strobus* ovules associated with fertilization and early embryo development. Can. J. For. Res. 17:408-412.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Zimmer, E.A., Rivin, C. and Walbot, V. 1981. A DNA isolation procedure suitable for most higher plant species. Plant Molec. Biol. Newslett. 2:93-96.

PRESERVATION OF TROPICAL PLANT MATERIAL FOR MOLECULAR ANALYSES

Stephen A. Harris and Julian Robinson
Sir Harold Mitchell Building, Department of Biological and Medical Sciences
University of St. Andrews,
St. Andrews, Fife KY16 9TH Scotland

SUMMARY

The preservation of DNA from tropical plants by desiccation was examined. Data from 25 leguminous taxa and one bombacaceous taxon were collected. These taxa yielded DNA, useful for restriction analysis, independent of the drying method used. Storage of legume DNA for up to 289 days at ambient temperature indicated that, over this period, there was no significant degradation of the nuclear DNA, but that there was significant degradation of chloroplast DNA sequences. The literature on DNA preservation is reviewed and the use of air-dried or corrugate-dried tropical material is advocated as this may lead to increased collaboration with tropical collectors at minimal expense.

INTRODUCTION

The material of choice in molecular systematic studies has been fresh, or freshly lyophilised, leaf tissue (Milligan, 1992). This means that many tropical taxa, especially woody groups, have been overlooked due to the potential lack of representation in botanical gardens, lack of seed set in isolated garden specimens and individuals, occurrence of seed recalcitrance (Chin and Roberts, 1980; Harrington, 1972; Roberts, 1973) and difficulties of cultivation. Even if material is available in botanical gardens or as seed, then the availability of multiple accessions is unlikely, unless the taxon of interest is commercially important. This is a serious drawback for workers interested in intraspecific and geographical variation, population structure and hybridization and/or introgression events. The growing use of population genetics in systematic studies means that adequately sampled material is essential, especially as there is now increasing evidence of intraspecific chloroplast DNA variation (Harris and Ingram, 1991; Lavin et al., 1991; Soltis et al., 1991).

Some early workers chose to collect fresh material in the field and air-freight it back to the laboratory on ice, e.g., *Lisianthius* (Gentianaceae; Sytsma and Schaal, 1985). However, for the majority of studies this is both expensive and impractical. Logistical and financial constraints have led some workers to consider the possibility of using alternative plant preservation methods for molecular analyses (Doyle and Dickson, 1987; Liston et al., 1990). Two basic approaches have been used: chemical preservation and dry preservation.

One of the first examinations of the effects of fluid preservation on DNA was that of Doyle and Dickson (1987), who studied the chemical preservation of *Solanum glutinosum* Dunal. (Solanaceae) leaves, showed that formalin-acetic acid-ethanol (FAA) Carnoy's fluid (ethanol: acetic acid, 3:1) and 10% brine solution did not preserve DNA for more than a week, though chloroform:methanol (4:3) did preserve DNA for three weeks. Pyle and Adams (1989) examined the effects of chemical preservatives on DNA preservation in *Spinacia* sp. (Chenopodiaceae). These workers showed that Carnoy's

fluid, 95% ethanol, glycerol, 3% sodium azide, Perfix preservative, pentachlorophenol/chloroform (1.5:98.5), 10% paraformaldehyde, 1% guanidine thiocyanate, 25% brine solution, 5% Chlorox, methanol:chloroform:propionic acid (1:1:1), 2% and 8% glutaraldehyde, 7.4% formaldehyde, 10% trichloroacetic acid, 0.5M sodium cacodylate and 10mM EDTA did not preserve DNA for seven days. Similarly, Adams (1990), using *Spinacia* sp. leaves, showed that phenol and 0.5M EDTA did not preserve DNA for seven days. However, copper oleate solution gave DNA fragments of 20 - 2 kb and 5% Chlorox solution gave DNA fragments of 50 - 20 kb, when extracted after seven days. Rogstad (1992) showed that a solution saturated with sodium chloride and hexadecyltrimethylammonium bromide (CTAB) was useful for preserving leaves suitable for subsequent DNA analyses in *Podophyllum peltatum* L. (Berberidaceae), *Polyalthia glauca* (Hassk.) Mueller (Annonaceae), *P. lateriflora* (Blume) King, *P. sclerophylla* Hook. fil & Thompson and *Taraxacum officinale* Wiggers (Asteraceae). Leaves preserved in this manner were useful after storage for one month at ambient temperature and after storage for at least one year at -20°C. Chemical preservatives have been recommended for the preservation of unicellular algae destined for DNA analysis (Stam et al., 1989).

A study of three dry fungicides (sulfur powder; Maneb Mn and Zn powder; napthalene) and a dry bactericide (streptomycin sulfate) on the preservation of *Spinacia* sp. leaves (Adams, 1990) has shown that these preservatives gave DNA fragments of 50 - 2 kb. However, Adams did not rule out the possibility that desiccation may have been as important as any biocidal action for DNA preservation.

Chemical preservation of field-collected material destined for molecular analyses is mostly impractical since the most effective chemicals (chloroform and CTAB) are very toxic. The difficulty of obtaining many of these chemicals inside tropical countries would reduce the participation, and highly desirable collaboration, of local field-based botanists. The supply of these chemicals to collaborators could be envisaged (e.g., Chase and Hills, 1991 with silica gel), but difficulties might be experienced with both the carriage of toxic materials and customs officials. In any case fluid-preserved materials would be both heavy and bulky and, therefore, difficult and costly to transport.

One answer to this problem would be to preserve material for molecular analyses, at least initially, in a dry state. This method has a number of advantages:- (i) the technique is easily undertaken and most tropical botanists are familiar with its use; (ii) voucher material is essential, therefore little additional work would be necessary to obtain material suitable for molecular studies; (iii) dried material can quickly, easily and cheaply be transported. The solution to the problem appears to exist. However three questions remain. Is dried material a suitable source of DNA? What are the effects of different drying regimes? How long can dried material be stored before it becomes useless for DNA analyses?

The first studies on the feasibility of DNA extraction from dried material came from studies of animals, e.g., the extinct quagga (Higuchi et al., 1984) and Egyptian mummies (Pääbo, 1985, 1989). One of the first studies to examine the feasibility of using dried plant material as a source of DNA was that of Rogers and Bendich (1985). Herbarium samples (23 to 118 years old) from seven taxa [*Elytrigia juncea* (L.) Nevski.; *Poa juncifolia* Schribn.; *Poa palustris* L.; Teosinte/maize cross; *Triticum aestivum* L.; *Zea mays* L.; *Vicia faba* L.] of the Poaceae and Fabaceae were studied. Measurable quantities of DNA were obtained in each case, but the average DNA size varied between 1.0 - 0.1 kb. More recent studies have shown that high molecular weight DNA (HMW-

DNA) can be extracted from dried material of many different woody and herbaceous genera in many different families (Table 1). These studies clearly show the feasibility of using dried material as a source of DNA.

Table 1. Families and genera from which high molecular weight DNA has been isolated, following desiccation and/or drying.

Family	Genus	Reference
Arecaceae	*Caryota*	Doyle and Dickson, 1987
Anacardiaceae	*Astronium*	Chase and Hills, 1991
	Schinus	Chase and Hills, 1991
Asteraceae	*Artemisia*	Liston et al., 1990
Brassicaceae	*Lepidium*	Liston et al., 1990
Caryophyllaceae	*Cerastium*	Liston et al., 1990
Chenopodiaceae	*Ceratoides*	Liston et al., 1990
Crassulaceae	*Rhodiola*	Liston et al., 1990
Cupressaceae	*Juniperus*	Pyle and Adams, 1989
Cyperaceae	*Carex*	Liston et al., 1990
Datiscaceae	*Datisca*	Liston et al., 1992
Ericaceae	*Arbutus*	Liston et al., 1990
Fabaceae	*Astragalus*	Liston et al., 1990
	Glycine	Doyle and Dickson, 1987
	Glycyrhiza	Liston et al., 1990
	Leucaena	Harris, submitted
	Piptadenia	Harris, submitted
Fagaceae	*Quercus*	Pyle and Adams, 1989
Gentianaceae	*Gentiana*	Liston et al., 1990
Haemodoraceae	*Anigozanthos*	Chase and Hills, 1991
Liliaceae	*Alstroemeria*	Chase and Hills, 1991
Magnoliaceae	*Talauma*	Chase and Hills, 1991
Malpighiaceae	*Dicella*	Chase and Hills, 1991
	Gaudichaudia	Chase and Hills, 1991
	Janusia	Chase and Hills, 1991
	Malpighia	Chase and Hills, 1991
	Mascagnia	Chase and Hills, 1991
Paeoniaceae	*Paeonia*	Liston et al., 1990
Plantaginaceae	*Plantago*	Liston et al., 1990
Poaceae	*Aeluropus*	Liston et al., 1990
Portulacaceae	*Claytonia*	Doyle and Dickson, 1987
Primulaceae	*Glaux*	Liston et al., 1990
Orchidaceae	*Corallorhiza*	Doyle and Dickson, 1987
	Epistephium	Chase and Hills, 1991
Rosaceae	*Potentilla*	Liston et al., 1990
Rubiaceae	*Coffea*	Harris, submitted
Scrophulariaceae	*Lagotis*	Liston et al., 1990
Solanaceae	*Solanum*	Doyle and Dickson, 1987
Trigoniaceae	*Trigonia*	Chase and Hills, 1991
Vochysiaceae	*Callisthene*	Harris, submitted
	Qualea	Harris, submitted
	Salvertia	Harris, submitted
	Vochysia	Harris, submitted

Four groups of drying regimes are available for the preservation of DNA: (i) chemical-drying, i.e. phosphorus pentoxide, anhydrous calcium sulfate (Drierite) and silica gel (Chase and Hills, 1991; Dally and Second, 1989; Liston et al., 1990; Pyle and Adams, 1989); (ii) slow drying, e.g., contact paper-drying and air-drying (Adams, 1990; Adams et al., 1992; Doyle and Dickson, 1987; Harris, submitted); (iii) rapid drying, e.g., corrugate-drying (Harris, submitted); (iv) microwave-drying (Adams, 1990; Adams et al., 1992). Few comparative studies have been conducted on the effects of different drying regimes (Adams, 1990; Adams et al., 1992; Harris, submitted). Chemical-drying, slow drying and rapid drying have been shown to be effective for the preservation of material suitable for DNA extraction from a wide range of temperate and tropical material. Adams's studies (Adams, 1990; Adams et al., 1992) have shown that three minutes microwave-drying of *Spinacia* leaves leads to DNA degradation after 24 hours. The limited amount of data available would seem to indicate that microwave-drying of leaves leads to rapid DNA degradation (Adams, 1990; Adams et al., 1992). The commonly used drying technique employed in the tropics of 'pickling' herbarium specimens in 70% ethanol, prior to drying at a later stage, appears to lead to rapid DNA degradation in *Solanum albidum* Dunal. (Doyle and Dickson, 1987).

The length of time that a dried sample can be stored before it becomes useless for molecular analysis depends on two factors: the molecular methods to be used in the study and the storage conditions of the dried sample. Methods based on restriction analysis (e.g., Palmer, 1986) or DNA fingerprinting (Nybom, 1991) require HMW-DNA for the best resolution; however, methods based on PCR amplification can utilize DNA samples which are much more degraded (Welsh and McClelland, 1990; Welsh et al., 1991). Adams and colleagues have shown that *Spinacia* and *Juniperus virginiana* L. (Cupressaceae) DNA, extracted from dried material, at least eight months old and stored at -20°C, remains intact (Adams, 1990; Adams et al., 1992). When stored at 37°C DNA degradation occurred, but was most obvious in *Magnolia grandiflora* Mill (Magnoliaceae) and *Quercus virginiana* L. (Fagaceae) leaves. Doyle and Dickson (1987) have shown that DNA degradation occurred in *Glycine tabacina* (Labill.) Benth. (Fabaceae) after two years.

Few studies have been conducted using field-collected tropical material (Chase and Hills, 1991; Harris, submitted) to examine the effects of storage time and drying method. In the present report we show that HMW-DNA can be extracted from legume material up to 289 days old and that the method of drying does not appear to affect the quality of extracted total DNA. Preliminary evidence is provided to suggest that differential degradation of DNA sequences may occur during storage of dried leaves at ambient temperature.

MATERIALS AND METHODS

Leaf material from *Chorisia speciosa* was collected in Argentina and leaf material from the Fabaceae was collected in Africa, Central America and Mexico (details of collections are available on request). All of the material studied (Table 2) was collected in the field as young leaf material, free from visible fungal and insect damage and dried by one of three methods: (i) contact-paper drying, consisted of placing fresh leaves between sheets of newsprint in a plant-press and changing the papers daily until the specimens were dry; (ii) Silica-drying, consisted of placing approximately 1 g of young leaves into a snap-top plastic bag with about 25 g of self-indicating silica gel according to the method of Liston et al. (1990); (iii) Corrugate-drying, involved sandwiching the

leaf material between two layers of blotting paper and separating adjacent samples with aluminum corrugates before the stacks were clamped and dried overnight above a kerosene burner. Methods of treatment for each of the DNA samples are shown in Table 1. After drying, samples were stored at ambient temperature in snap-top plastic bags with a few crystals of silica gel.

Table 2. Quality of DNA extracted from dried leaf material of 25 Fabaceae taxa and one Bombacaceae species versus method of drying and time of storage.

Taxon	Drying[a]	Age[b]	Size[c]
Bombacaceae			
Chorisia speciosa St.-Hil.	A	57	G/F
Fabaceae			
Acacia galpinii Burtt Davy	S	134	G
A. hebeclada DC subsp. *chobiensis* (O.B. Miller) Schreiberi	S	126	G
A. rehmanniana Schinz.	S	115	G
A. exuvialis Verdoorn	S	115	G
A. nigrescens Oliv.	S	115	G
A. mellifera (Vahl.) Benth. subsp. *detinens* (Burch.) Brenan	S	125	G
A. burkei Benth.	S	115	G
A. caffra (Thunb.) Willd.	S	115	G
A. chariessa Milne Redh.	S	115	G
A. leuderitzii Engl. var. *leuderitzii*	S	115	G
A. pringlei Rose	C	277	G
A. amentacea DC.	C	287	G
A. mammifera Schlect.	C	289	G
A. schaffneri (Wats.) Hermann	C	279	G
A. subangulata Rose	C	289	G
Albizia versicolor Welw. ex Oliver	S	115	G
A. harveyi Fourn.	S	115	G
A. schimpera Fourn.	S	115	G
Leucaena "confertiflora" Zarate ined.	A/C/S	23	G[d]
L. cuspidata Standley	A/C/S	38	G[d]
L. diversifolia (Schlecht.) Benth. s.l.	A/C/S	21	G[d]
L. diversifolia (Schlecht.) Benth. subsp. *stenocarpa* (Urban) Zarate comb. ined.	C	234	G
L. leucocephala (Lam.) de Wit subsp. *glabrata* (Rose) Zarate x *L. esculenta* (Mocc. & Sess. ex DC.) Benth. subsp. *esculenta*	A/S	7	G[d]
Leucaena sp.	C		G[d]
Piptadenia viridiflora Benth.	S	19	G[d]

[a] A = Contact-paper drying (i.e. frequent changing of drying papers); C = Corrugate drying (i.e. overnight on a kerosene stove); S = Silica gel drying.
[b] Date between harvesting and DNA extraction.
[c] Adams (1990) classification of fragment sizes. Good (G): mostly 50 - 20 kb; Fair (F): 20 - 2 Kb.
[d] from Harris (submitted).

Intact, total DNA was extracted from leaf material of individual plants according to the method of Doyle and Doyle (1987), but with the following modifications; 1% PVP-40T was added to the 2 x CTAB extraction buffer and the mixture incubated for 30 minutes at 65°C. For *Acacia* and *Albizia* samples, ß-mercaptoethanol was excluded from the extraction buffer. The extraction buffer: dry weight (dw) ratio was greater than 25 ml/g dw. Samples were purified on caesium chloride density gradients containing 0.75 g/ml caesium chloride and 100 µg/ml ethidium bromide and centrifuged overnight at 20°C at 40,000 rpm (Sorvall OTD65B centrifuge with T865.1 rotor; Maniatis et al., 1982).

Estimates of DNA size were made by visual estimation of ethidium bromide fluorescence of DNA following electrophoresis of samples on 0.8 % agarose gels at 40 mA for 4 - 6 hours in a 1 x SEB gel buffer [0.04 M Tris-HCl, 0.02 M sodium acetate, 1 mM disodium EDTA, pH 7.85]. An intact calf thymus DNA standard of known concentration was included on each gel.

To test digestion of the DNA samples and their use in molecular studies, aliquots of the DNA samples were cut with *Bam*HI, *Hin*DIII and *Eco*RI, and the fragments separated on 1 % agarose gels at 60 mA overnight. DNA fragments were blotted on to Amersham Hybond N or BDH nylon membrane and baked for 2 hours at 80°C. Filters were probed with chloroplast DNA (cpDNA) probes from the mung bean library (Palmer and Thompson, 1981) and a nuclear ribosomal DNA fragment from wheat DNA (Gerlach and Bedbrook, 1979). Probes (60 ng) were labeled by the random primer method (Feinberg & Vogelstein 1983) using ^{32}P-dCTP (3000 Ci/mM, 10 µCi). Hybridization took place overnight at 65° C in Buffer III [0.6 M sodium chloride, 10 mM PIPES (pH 6.8), 1 mM disodium-EDTA (pH 8.5), 10 x Denhardt's solution]. Filters probed with cpDNA probes were washed according to the conditions of Palmer (1986) and ribosomal DNA-probed filters were washed as follows: a brief rinse in 2 x SSC at room temperature followed by two 20 minute washes in 1 x SSC + 0.1 % SDS at 65°C.

RESULTS AND DISCUSSION

Dried leaf material from 25 woody Mimosoid leguminous taxa and one bmbacaceous taxon have been examined as sources of DNA for molecular studies (Table 1). In all of the mimosoid legume taxa examined HMW-DNA was successfully extracted, though the relative yields varied between accessions. All samples were successfully digested with restriction enzymes and probed with mung bean chloroplast DNA probes. In the case of *Chorisia speciosa* HMW-DNA was obtained, but there was some degradation. The degradation observed in *Chorisia speciosa* may reflect the presence of secondary plant metabolites in the Bombacaceae; unfortunately we could not test fresh material as this was unavailable to us. Previous studies have shown that difficulties may be experienced in the extraction of DNA from woody taxa (Adams, 1990; Adams et al., 1992; Chase and Hills, 1991; Liston et al., 1990; Pyle and Adams, 1989). Despite the partial degradation, the DNA sample was readily digested with restriction enzymes and produced distinct bands when probed with nuclear ribosomal DNA sequences. DNA samples from *Chorisia* were also successfully amplified using PCR primers (data not shown).

The utility of drying as a method of DNA preservation and the ease of DNA extraction cannot be predicted in advance as the quality of the extracted DNA appears to vary from taxon to taxon (Chase and Hills, 1991; Doyle and Dickson, 1987; Harris,

submitted; Rogstad, 1992). Some families are apparently more amenable to DNA extraction from dried material, e.g., Fabaceae (Doyle and Dickson, 1987; Harris, submitted) and Vochysiaceae (Harris, submitted) than others, e.g., Malpighiaceae (Chase and Hills, 1991) and Fagaceae (Adams et al., 1992). It is therefore very important that before a major collecting effort is made that a method of DNA extraction is perfected and its application to dried material determined. However, in general dried plant material does appear to be a useful method of preservation for molecular studies of tropical taxa. Comparison of the quality of DNA samples from leaves dried by silica gel, rapid and slow drying showed no visible difference in the quality of total DNA extracts. Any of these three methods appear to be useful for tropical legume material, although the utility of the three methods in other plant families has yet to be tested.

The ability to use leaves dried in even very crude ways for molecular analyses (e.g.,. Doyle and Dickson, 1987) means that fruitful collaboration with botanists from tropical countries can be established. Neither special chemicals nor techniques are needed; the techniques of contact-paper and corrugate drying are widely used by field botanists. The most important factor is the quality of the plant material collected. This should be as young as possible and free from fungal, viral and insect damage. Once collected and dried, leaf material can easily be dispatched by post in paper envelopes or snap-top plastic bags with a few crystals of silica gel.

Leaf samples in this study were all stored at ambient temperature, which varied from greater than 30°C when the material was in the tropics to less than 10°C when it was in the U.K. Material used in this study was from seven to 289 days old when DNA was extracted. Using DNA fluorescence, as a measure of DNA size and concentration, showed little difference between DNA extracted from material stored for 7 days at ambient temperature and DNA extracted from material stored for 289 days at ambient temperature. However the situation regarding DNA degradation at ambient temperature may be more complex. Estimates of total DNA concentration were made by visual inspection of ethidium bromide fluorescence compared to a standard of known concentration. When fragment intensities of restriction digested *Acacia* and *Albizia* DNAs, isolated from dried and fresh material, were probed with cpDNA sequences it was found that the cpDNA concentrations in dried material had been underestimated compared to fresh material (Figure 1). This suggests that there has been selective degradation of the chloroplast genome. Silica gel-dried leaves (mean of 115 days old) showed a 30% to 50% reduction in cpDNA band intensity compared to fresh material and corrugate dried leaves (mean of 284 days old) showed a 40% to 70% reduction in cpDNA band intensity compared to fresh material. Similar results were observed for *Leucaena* cpDNA, but when nuclear DNA was surveyed with a ribosomal DNA probe no differences were observed between fresh and dried material. In the case of *Leucaena* the observed effect appears to be due to aging rather than the method of drying, since no differences were found between DNA samples extracted from corrugate-dried, contact-paper dried or silica gel dried leaves of similar age. It would appear that cpDNA is lost on storage of dried leaves at ambient temperature. This has some important implications for the use of dried material as it would appear different genomes, and possibly sequences, show differential stability to prolonged storage at ambient temperature.

Long term storage of dried material at ambient temperature appears to lead to general degradation (Adams, 1990; Adams et al., 1992) and degradation of specific genomes. Dried material is, therefore, best stored at -20°C (or -70°C) for longer periods of time. Periods of up to 38 days storage at ambient temperature do not appear

Figure 1. Differences in the relative amounts of chloroplast DNA sequences between *Acacia* DNA isolated from fresh (lanes 1, 5, 6, 7, 11), silica gel-dried (lanes 2, 3, 4, 8, 9) and corrugate-dried (lane 10). *Bam*HI-digested total DNA probed with mung bean chloroplast DNA probe MB3. Approximately 500 ng total DNA (as determined by visual inspection) loaded per lane. Lane 1: *A. polycantha* Willd. ssp. *campylacantha* (Hochst. ex A. Rich.) Brenan; lane 2: *A. caffra*; lane 3: *A. nigrescens*; lane 4: *A. burkei;* lane 5: *A. senegal* (L.) Willd. var. *leiorhachis* Brenan; lanes 6 and 7: *A. senegal* (L.) Willd. var. *senegal;* lane 8: *A. mellifera;* lane 9: *A. chariessa;* lane 10: *A. mammifera;* lane 11: *A. angustissima* (Mill.) Kuntze.

to effect degradation (Harris, submitted), which should be long enough for most field collectors to transfer their collections to sub-zero temperatures.

The advantages of using preserved material compared to fresh material for molecular studies have already been pointed out. These include: (i) access to a much wider range of material; (ii) closer collaboration with colleagues in tropical countries; (iii) ability to use vegetative material, rather than the need to collect seed, so collection expeditions need not necessarily coincide with fruit set; (iv) analysis of sterile plants, e.g., a sterile hybrid of *Leucaena* (Hughes and Harris, unpublished data). These factors are enhanced when dried material is used since the techniques are easily applied and are not labor intensive.

Dried material, as a source of DNA, may lead to a new use for herbarium duplicates (Harris, submitted). The effort associated with collecting an additional duplicate for molecular use, which is young and free from fungal, viral and insect damage, is negligible compared to the effort and logistical support required to collect in the first instance. Previously collected duplicates are likely to be unsatisfactory for restriction analyses due to DNA degradation after long term storage, but this does not negate their use in PCR based studies. Current collection strategies should be reconsidered. New collecting expeditions ought to consider the collection of duplicates for molecular analysis and the storage of this material at sub-zero temperatures.

ACKNOWLEDGEMENTS

We would like to thank Marta Bianchi, Chris Fagg, Peter Gibbs and Colin Hughes who have supplied us with most of the dried leaf material. J. J. Doyle and M. O'Dell kindly supplied the probes used. We would like to thank David Curnow and Ruth Ingram for their very valuable comments on a early draft of this paper. Funding for this study has been provided by the ODA Forestry Research Programme (R.4727) and a University of St. Andrews Research Studentship to JR.

REFERENCES

Adams, R.P. 1990. The preservation of Chihuahuan plant genomes through *in vitro* biotechnology: DNA Bank-Net, a genetic insurance policy, *in:* "Third Symposium on Resources of the Chihuahuan Desert Region," A.M. Powell, R.R. Hollander, J.C. Barlow, W.B. McGillivray and D.J. Schmidly, eds., Chihuahuan Desert Research Inst., Printech, Lubbock, TX.

Adams, R.P., Do, N. and Ge-Lin, C. 1992. Preservation of DNA in plant specimens from tropical species by desiccation, *in:* "Conservation of Plant Genes: DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

Chase, M.W. and Hills, H.H. 1991. Silica gel: An ideal material for field preservation of leaf samples for DNA studies. Taxon 40: 215.

Chin, H.F. and Roberts, E.H. 1980. "Recalcitrant Crop Seeds." Tropical Press, Kuala Lumpur.

Dally, A.M. and Second, G. 1989. Chloroplast DNA isolation from higher plants: an improved non-aqueous method. Plant Mol. Biol. Rep. 7:135.

Doyle, J.J. and Dickson, E.E. 1987. Preservation of plant samples for DNA restriction endonuclease analysis. Taxon 36:715.

Doyle, J.J. and Doyle, J.L. 1987. A rapid isolation procedure for small amounts of fresh leaf tissue. Phytochem. Bull. 19:11.

Feinberg, A.P. and Vogelstein, B. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6.

Gerlach, W.L. and Bedbrook, J.R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. Nucleic Acids Res. 7:1869.

Harrington, J.F. 1972. Seed storage and longevity, *in:* "Seed Biology. Volume 3," T.T. Kozlowski, ed., Academic Press, New York.

Harris, S.A. and Ingram, R. 1991. Chloroplast DNA and biosystematics: The effects of intraspecific diversity and plastid transmission, Taxon 40:393.

Harris, S.A. 1993. DNA analysis of tropical plant species: An assessment of different drying methods, Pl. Syst. Evol. (Submitted).

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A. and Wilson, A.C. 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312:282.

Lavin, M., Mathews, S. and Hughes, C. 1991. Chloroplast DNA variation in *Gliricidia sepium* (Leguminosae): Intraspecific phylogeny and tokogeny. Am. J. Bot. 78:1576.

Liston, A., Rieseberg, L.H., Adams, R.P., Do, N. and Zhu, G.-I. 1990. A method for collecting dried plant specimens for DNA and isozyme analyses and the results of a field test in Xinjiang, China. Ann. Mo. Bot. Gard. 77:859.

Liston, A., Rieseberg, L.H. and Hanson, M.A. 1992. Geographic partitioning of chloroplast DNA variation in the genus *Datisca* (Datiscaceae). Pl. Syst. Evol. 181:121.

Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. "Molecular Cloning: a Laboratory Manual." Cold Spring Harbor Publications, Cold Spring Harbor.

Milligan, B.G. 1992. Plant DNA isolation, *in:* "Molecular Genetic Analysis of Populations. A Practical Approach," A.R. Hoelzel, ed., IRL Press, Oxford.

Nybom, H. 1991. Applications of DNA fingerprinting in plant breeding, *in:* "DNA Fingerprinting: Approaches and Applications," T. Burke, G. Dolf, A.J. Jeffreys, and R. Wolff, eds., Birkhauser Verlag, Basel.

Pääbo, S. 1985. Molecular cloning of ancient Egyptian mummy DNA. Nature 314:644.

Pääbo, S. 1989. Ancient DNA: Extraction, characterization, molecular cloning and enzymatic digestion. Proc. Natl. Acad. Sci. U.S.A. 86:1939.

Palmer, J.D. 1986. Isolation and structural analysis of chloroplast DNA. Meth. Enzym. 118:167.

Palmer, J.D. and Thompson, W.F. 1981. Rearrangements in the chloroplast genomes of mung bean and pea. Proc. Natl. Acad. Sci. U.S.A. 78:5533.

Pyle, M.M. and Adams, R.P. 1989. *In situ* preservation of DNA in plant specimens, Taxon 38:576.

Roberts, E.H. 1973. Predicting the storage life of seeds. Seed Sci. Technol. 1:499.

Rogers, S.O. and Bendich, A.J. 1985. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Pl. Mol. Biol. 5:69.

Rogstad, S.H. 1992. Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analyses. Taxon 41:701.

Soltis, D.E., Soltis, P.S. and Milligan, B.G. 1991. Intraspecific chloroplast DNA variation: systematic and phylogenetic implications, *in:* "Molecular Systematics of Plants," P.S. Soltis, D. E. Soltis and J. J. Doyle, eds., Chapman and Hall, London.

Stam, W.T., Lewin, R.A. and Cheng, L. 1989. DNA conservation for taxonomic studies. J. Phycol. 25:615.

Sytsma, K.J. and Schaal, B.A. 1985. Phylogenetics of the *Lisianthius skinneri* (Gentianaceae) species complex in Panama utilizing DNA restriction fragment analysis. Evol. 39:594.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213.

Welsh, J., Petersen, C. and McClelland, M. 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucleic Acids Res. 19:303.

DNA ARCHIVAL STORAGE AND RETRIEVAL SYSTEMS

Elliott P. Dawson[1], Kan Wang[2], Shu-Ping Jiao[2],
James R. Harris[1] and James R. Hudson[3]
[1]BioVentures, Inc., P. O. Box 2561, Murfreesboro, Tennessee 37133-2561, USA
[2]ICI Seeds, Slater, Iowa
[3]Research Genetics, Huntsville, Alabama

SUMMARY

The use of the GeneReleaser is investigated using transgenic maize and found to be equivalent to a mini-prep DNA extraction for the maize utilized. GeneKeeper, designed for interim collection and storage of DNA at ambient temperatures, was investigated using 12 and 28 day old maize plants. After a simple water/microwave treatment, direct PCR was successful on these samples. The GeneKeeper may provide a method of specimen collection, archival storage and retrieval for DNA studies of rare and endangered plant specimens from regions of the world that are remote and without facilities for sample processing.

INTRODUCTION

To say that the polymerase chain reaction (PCR) has become a technique of choice for molecular biologists since its invention by Kary Mullis in 1985 is to state the obvious. In order to successfully perform PCR, DNA must be obtained in suitable form. The DNA must be free of any components which inhibit PCR and the DNA must contain the sequence of interest in undegraded form and be free of contaminating DNA. Obtaining DNA of suitable quality for PCR is fundamentally dependent on the source material. The difficulty of obtaining DNA of adequate quality for PCR ranges from being absurdly simple to Herculean. Because of their complex biochemistry and the production of secondary metabolic products which inhibit polymerase activity, plant materials represent some of the most challenging tissues from which to obtain DNA suitable for use in PCR.

The application of molecular biological techniques to the selection, propagation and modification of plants of agricultural interest has steadily grown. A number of PCR based techniques have been developed and applied to these processes. In many instances it is desirable and preferred to analyze a large number of specimen samples. However, obtaining DNA suitable for amplification by PCR from large numbers of samples presents formidable logistical problems. Foremost among these problems are the labor requirements associated with sample collection and the proper preparation of DNA for amplification. The economic costs of labor and the associated costs of reagents, equipment and other resources required for sample preparation are also significant. The combination of logistical and economic constraints impede the application of PCR to the analysis of plants of agricultural and other significance. Today hundreds of specimens can be analyzed and screened by PCR, but in many instances several thousand to several million assays are desired to both assure, enhance and accelerate plant selection and breeding programs.

In order to diminish both the effort and costs associated with sample preparation and collection of DNA for PCR and other molecular biology techniques, two products have been developed by a joint research effort between BioVentures, Inc.

(Murfreesboro, TN) and Research Genetics (Huntsville, AL). The first is GeneReleaser™ and the second is GeneKeeper™. GeneReleaser is a proprietary reagent which releases DNA and RNA from tissues, cells, organelles, microorganisms and the like. The release of nucleic acids is accomplished in a matter of minutes and nucleic acids have been obtained and amplified by PCR by treatment of specimens such as whole blood, mouse tail and spleen. Clinically significant infectious organisms such as HIV and *Mycobacterium tuberculosis* have been detected directly in whole blood and sputum using DNA obtained by GeneReleaser treatment (data not shown). Lysis and release of nucleic acids is typically accomplished using 1µl of specimen and 20µl of GeneReleaser in a standard amplification tube on a thermocycler. Recently a microwave sample treatment with GeneReleaser has been described (BioVentures, 1992). GeneReleaser sequesters cell lysis products and preservatives that inhibit polymerases or degrade nucleic acids. GeneKeeper is a device for the collection, storage and retrieval of nucleic acids and other substances for analysis. The devices employed in the present study contain ten application zones. Samples are applied to the zones, fixed in ethanol and are air dried. DNA is retrieved for analysis from the zones and is amplified by PCR or other techniques. HLA analysis has been successfully performed from whole blood collected on GeneKeeper devices and stored for one year prior to analysis.

Recently, several DNA isolation methods intended to overcome some of the problems associated with plant materials have been reported. The simplest of them is one in which fresh tobacco leaf or root tissues are placed directly into the PCR reaction buffer to provide a source of DNA for PCR amplification (Berthomieu and Mayer, 1991). Other methods (Edwards et al., 1991; Langridge et al., 1991; Oard and Dronavalli, 1992; Tai and Tanksley, 1990), although reliable, are still too complicated to match the ease of PCR for the analysis of large numbers of samples in a relatively short period of time. Furthermore, most of the procedures are multi-step processes requiring several reagent solutions and multiple manipulations or transfers which increase the possibility of cross-contamination. Here we report on simple and rapid preparation of plant DNA from maize for PCR analysis by using GeneReleaser and GeneKeeper.

EXPERIMENTAL METHODS

GeneReleaser Processed Specimens

Three varieties of maize were grown at ICI Seeds Research Group (Slater, IA). Two of the varieties (A and B) contained transgenic DNA sequences and the third variety (BE81) was free of any transgenic DNA sequences. Maize leaf samples were collected from each of the three varieties by punching a portion of the leaf into a standard 1.5 ml "snap cap" centrifuge tube using the cap as the punch. Paired replicates of the maize leaf materials were processed by the method of Edwards et al., 1991, and by treatment with GeneReleaser. The GeneReleaser processed specimens were prepared by first grinding the leaf punch in the 1.5ml collection tube with a disposable plastic pestle until fluid was observed to be extruded from the leaf tissue. Fifty µl of sterile distilled water was added and 1 µl of this mixture was transferred to a standard 0.5ml amplification tube. Twenty µl of well mixed GeneReleaser was added to each amplification tube and vortexed briefly to mix. The tubes were then overlaid with mineral oil, placed in a suitable rack and placed in a microwave oven (Amana, 600 Watts). The tubes were then microwaved on HIGH setting for no longer than 6 minutes. The tubes were then placed in the block of a thermocycler preheated to 85°C and equibrated for 5 minuses. Eighty µl of a 1.25x PCR master mix containing all

components for amplification except target DNA was added to each of the tubes. Amplification was performed using conditions previously optimized for the amplification of purified DNA from the transgenic maize varieties. The PCR products were assayed by standard agarose gel electrophoresis, visualized by ethidium bromide staining and photographed. A set of water controls was processed in parallel with the samples.

GeneKeeper Processed Specimens

Two varieties of maize were grown in the laboratory. Leaf material from young plants (12 and 28 days of growth) was collected on the GeneKeeper device by folding the leaf around the edge of the GeneKeeper and vigorously rubbing the leaf against the device until sufficient tissue had been abraded from the leaf to color the collection zones green. The devices were then dipped into 95% ethanol, removed and air dried under ambient conditions. Specimens collected on the devices were stored at room temperature until processed.

Retrieval of DNA from the devices for amplification was performed by punching out the sample zone into a standard 0.5ml PCR tube with a 3/16 inch plastic disposable dowel. Twenty µl of sterile deionized water was added to the tube, followed by vortexing for 30 seconds to disrupt the zone material. The tubes were overlaid with mineral oil, closed and placed in a suitable rack. The rack was placed in the center of a microwave oven (Panasonic, 900 Watts) and were microwaved for 5 minutes on the HIGH setting. A set of three serial 1 to 5x dilutions were prepared by taking 1µl of the supernatant from the microwave treated material and diluting with 1x TE giving 1:5, 1:25 and 1:125 dilutions of the microwaved material. One µl of the undiluted and 1µl of the three serial dilutions were each added to separate tubes and 19 µl of sterile water was added to each. The tubes were then overlaid with mineral oil. The tubes were then transferred to the preheated block of a thermocycler (Perkin Elmer, Model 480) at 80°C for 5 minutes. Eighty µl of a 1.25x PCR master mix containing all components for amplification except target DNA was added to each of the tubes. Amplification was performed using conditions previously optimized for the amplification of purified DNA from the maize varieties. The PCR products were assayed by standard agarose gel electrophoresis, visualized by ethidium bromide staining and photographed. A set of water controls was processed in parallel with the samples.

RESULTS

The samples from the A and B transgenic maize varieties and BE81 prepared with GeneReleaser or the method of Edwards et al. (1991) gave comparable results for the amplification of the transgene sequences. Both the A and B transgenic varieties were found to yield the expected PCR products when processed by either method. The nontransgene containing variety BE81 failed to give amplification with transgene specific primers. Figure 1 is a photograph of an ethidium bromide gel which shows comparative results for the two methods of specimen processing.

GeneKeeper collected specimens gave expected bands for primers which amplify a sequence common to all maize strains at dilutions between 1:25 and 1:125 for 12 day old leaf while the 28 day leaf gave the expected product regardless of dilution over the range studied. In no case did the undiluted material provide amplification. The water only controls gave no amplification with either undiluted material of with any of the dilutions. The results of a typical amplification are illustrated in Figure 2. The GeneKeeper device employed in these experiments is illustrated in Figure 3.

Figure 1. Comparison of GeneReleaser (lanes 1-8) and a multi-step miniprep (lanes 9-10; Edwards, et al., 1991). Lanes 1, 5, and 9 are from transgenic maize A; lanes 2 and 6, transgenic maize B; lanes 3 and 7, control plant BE81; lanes 4, 8, and 10 are water controls.

DISCUSSION AND CONCLUSIONS

In the experiments with GeneReleaser, appropriate bands were observed for each transgenic maize variety studied. Use of an appropriate quantity of leaf homogenate in conjunction with GeneReleaser, when compared to the water controls, indicates that the sequestering action of the reagent permits the rapid processing and amplification of target DNA sequences from transgenic maize plants. Comparable results for the amplification of the transgenic sequence between the GeneReleaser treated specimens and those processed by the method of Edwards et al. (1991) demonstrate equivalent results. Unlike the method of Edwards et al. (1991), there is no need to perform a precipitation of DNA or for centrifugation by employing GeneReleaser. The use of GeneReleaser for the preparation of maize leaf DNA for PCR is not as simple as placing leaf materials directly in the reaction mixture as done by Berthomieu and Mayer (1991), but their method proved to be unsatisfactory for maize. Because GeneReleaser can sequester many of the PCR inhibitors found in plant materials, its use affords a very

Figure 2. PCR results from GeneKeeper treated maize leaf DNAs. Lanes 1-3 are DNA from 12 day leaves, diluted to 1:5; 1:25; and 1:125 concentrations. Lanes 4-6 and 8 are water only preps from 28 day leaves run as: neat; 1:5; 1:25; and 1:125 dilutions. Lane 7 is marker DNA. Lanes 9-12 are DNA from 28 day old leaves, run as: neat; 1:5; 1:25: 1:125 concentrations in PCR.

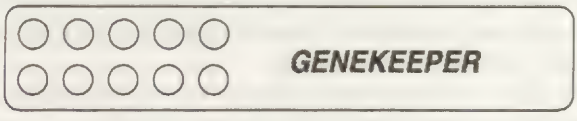


Figure 3. A GeneKeeper device with ten sample zones.

sequester many of the PCR inhibitors found in plant materials, its use affords a very simple method for the amplification of DNA from a broad variety of plant sources.

The experiments using GeneKeeper with maize leaf indicate that amplification can be successfully performed on maize leaf collected on the devices and stored for brief periods of time under ambient conditions. Comparison of the results with the 12 day old maize leaf versus the 28 day old leaf material indicates that the younger leaf material may contain inhibitors of PCR present in the younger leaf and absent in the older leaf or at higher concentrations in the younger leaf than in the older leaf. Comparison of the water controls to the GeneKeeper processed maize indicates that the GeneKeeper can prevent inhibition of PCR by these compounds although to a lesser degree with the younger leaf material than with the older leaf material.

Classic methods for the preparation of DNA from plant materials provide DNA

of sufficient quality for PCR. However, such methods are particularly cumbersome, especially if large numbers of specimens are to be processed for analysis. Classic methods of DNA isolation from plants are further encumbered by requiring in many instances the use of liquid nitrogen, organic extractions, gradient ultracentrifugation, DNA precipitation and relatively large quantities of plant material. With the advent of PCR, recent reports have appeared in the literature which describe methods which facilitate the processing of larger numbers of specimens for PCR. These methods, in addition to those previously described in the introduction may also require the use of liquid nitrogen, enzymatic digestion, extraction with organic solvents and precipitation of DNA (see for example: Brunel, 1992; Deragon and Landry, 1992; Junghans and Metzlaff, 1990; McGarvey and Kaper, 1991).

The disadvantages of many of the present methods for the preparation of plant DNA isolation methods for PCR and other molecular biology techniques are that in varying degrees they are labor intensive, process limited numbers of specimens, require fresh or frozen specimens, require modest to complete laboratory facilities, employ toxic or hazardous organic solvents and require skilled labor. If large quantities of a specimen are required, plants will be of greater maturity and may be entirely consumed in the analytical process. GeneReleaser overcomes many of these disadvantages by entirely avoiding many of the requirements imposed by other techniques for isolating plant DNA for PCR.

GeneKeeper devices were developed to accommodate the needs for sample collection, storage and retrieval for PCR. The device was developed to permit field collection by unskilled or semiskilled personnel requiring minimal skill for its use. Further it was desired to have a device that could be applied to the collection of a broad array of specimen types ranging from whole blood and other clinically relevant body fluids to plant materials. The devices were designed to survive the rigors of transport from remote regions and storage under ambient conditions without the requirement for refrigeration or freezing. The devices are designed so that multiple analyses can be performed on a single specimen at either different locations or at different times. The recovery of DNA for PCR from the devices is facile. Because material can be stored under ambient conditions for extended period of time the devices represent a means of archiving materials for recovery and analysis.

While this study focused on applications to PCR of maize DNA, other studies are in progress to expand the applications to other plants of agricultural significance. In the context of the present meeting, GeneKeeper may provide a method of specimen collection, archival storage and retrieval for DNA studies of rare and endangered plant specimens from regions of the world that are remote and without facilities for sample processing.

REFERENCES

Berthomieu, P. and Mayer, C. 1991. Direct amplification of plant genomic DNA from leaf and root pieces using PCR. Plant Mol. Biol. 17:555.

Brunel, D., 1992. An alternative, rapid method of plant DNA extraction for PCR analysis. Nucleic Acids Res. 20:4676.

Deragon, J.M. and Landry, B.S. 1992. RAPD and other PCR-based analysis of plant genomes using DNA extracted from small leaf disks. PCR Meth. and Appl. 1:175.

Edwards, K., Johnstone, C. and Thomson, C. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19:1349.

Junghans, H. and Metzlaff, M. 1990. A simple and rapid method for the preparation of total plant DNA. Biotechniques 8:176.

Langridge, U., Schwall, M. and Langridge, P. 1991. Squashes of plant tissue as substrate for PCR. Nucleic Acids Res. 19:6954.

McGarvey, P. and Kaper, J.M. 1991. A simple and rapid method for screening transgenic plants using the PCR. Biotechniques 11:428.

Mullis, K.B. and Faloona, F.A. 1987. Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. Methods Enzymol. 155:335.

Oard, J.H. and Dronavalli, S. 1992. Rapid isolation of rice and maize DNA analysis by random-primer PCR. Plant Mol. Biol. Reptr. 10:236.

Tai, T.H. and Tanksley, S.D. 1990. A rapid and inexpensive method for the isolation of total DNA from dehydrated plant tissue. Plant Mol. Biol. Reptr. 8:297.

RECENT ADVANCES IN THE USE OF PCR BASED TECHNOLOGY FOR DNA FINGERPRINTING IN PLANTS

Stephen Smith
Pioneer Hi-Bred International Inc., Plant Breeding Division
Department of Technology Support, 7250 NW 62nd Avenue, P.O. Box 1004
Johnston, Iowa 50131, USA

SUMMARY

Individual inbred lines and varieties of many crop species can be uniquely identified or "fingerprinted" by molecular markers. Fingerprint data must be reliably and repeatedly generated. In plants, and unlike humans, genetic distance between inbreds or varieties is an important factor. A finished inbred can be expected to still show a low level of segregation. Classical breeding practices, now augmented with molecular marker data, can quickly lead to the breeding or selection of inbreds or varieties that are closely similar to existing varieties. Transformation can achieve the same ends. Random primer profiling methods can, with relative ease and extreme rapidity, identify differences between extremely closely similar and related genotypes. Also, not all of these genotypic differences are reflected as phenotypic or agronomic differences. Therefore, it is necessary to define a boundary of protection with a prescribed distance around the focal point of the descriptor or profile. New Polymerase Chain Reaction (PCR) based profiling methodologies are rapidly appearing. Many of these methods may have practical application in cultivar identification provided the nature of plant "fingerprints" and the criteria necessary to support plant breeding are understood and adhered to.

INTRODUCTION

Individual inbred lines and varieties of many cultivated crop species can be uniquely identified by one or more sets of molecular markers. Individual segregating progeny of breeding populations can likewise be characterized. Initially, protein based methods such as seed storage proteins or isoenzymes were utilized. More recently, DNA based methods such as Restriction Fragment Length Polymorphisms (RFLPs) have been implemented. Most recently, methods based upon various DNA amplification systems using Polymerase Chain Reaction (PCR) thermocycling have been applied to individual plant and variety identification.

Applications of "Fingerprints" in Plant Breeding

Individual identification or "fingerprinting" in cultivated crop species has application in three broad areas. First, in the genetic identification of seed lot pedigrees and germplasm purity. The provision of genetically pure seedlots to the farmer that are of assured performance and grain quality attributes allows fulfillment of the effort invested in breeding and seed production. Second, discriminative and reliable descriptors characterize varieties efficiently and precisely, thereby providing an improved means to protect Plant Breeders' Rights (PBR). Therefore, varietal "fingerprints" can assist in creating an environment for the continued funding of plant breeding, genetic research and germplasm conservation. Third, molecular marker profiles can help plant breeders comprehend, organize and more effectively manage genetic resources. Molecular marker data can identify complementary pools of genetic resources and indicate at least

some of the chromosomal regions that affect expression of certain agronomically important traits. Therefore, molecular marker data can help provide more efficient selection, not only of parents for the making of breeding populations, but also of their agronomically superior progeny.

The Character of "Fingerprints" in Plants

The term "fingerprinting" has been applied to individual plant variety identification because, as in humans, techniques are available, at least for many species, that can repeatably discriminate among different varieties. Nonetheless, there are some differences between humans and cultivated varieties in respect of the concept and application of "fingerprints". These are important to recognize. First, it is possible, and indeed often part of routine breeding practice, to derive varieties that can be genetically extremely similar to a pre-existing variety. For example, classical backcrossing techniques or emergent transformation technologies can result in varieties that differ by as little as a single gene from an existing variety. Second, an inbred line or variety converges toward homozygosity at each generation of inbreeding. However, it is the inbred line, variety or F_1 hybrid that is profiled, not a specific individual plant. Even at the F_8 level, with single seed descent, there will remain a low level of residual heterozygosity. Third, it is quite possible to produce F_1 hybrids that are genetically very similar. Indeed some could be identical through the use by different seed producers or sales organizations of parental inbred lines and combinations thereof that are publicly or commercially widely available to the industry. In circumstances where inbreds can be discriminated, similarity of fingerprints among hybrids should not be taken as evidence of a lack of fingerprinting ability. Rather it is evidence of the relative lack of genetic diversity or degree of genetic identity among the hybrids.

All of these features that relate to crop varietal fingerprinting mean that it is useful and indeed necessary to consider the quantum of genetic (or genomic) differences which separate one variety from another. This "minimum distance" then serves to prescribe a boundary of protection in respect of PBR. Consequently, breeders are less concerned with discriminational ability per se than they are with the ability of fingerprint data to provide reliably useful measures of genetic distances between varieties. This is a demand not usually made upon human DNA fingerprints.

MOLECULAR MARKER SYSTEMS

From Isoenzymes to DNA Analysis Using Restriction Fragment Length Polymorphisms (RFLPs)

Molecular markers have been used for more than a decade in widespread routine support of plant breeding and seed production. Isoenzymes and seed storage proteins now enter their 13th year in providing routine validation of inbred and hybrid genetic identity and purity. Subsequently, more numerous loci were made available to discriminate among genotypes by the DNA based RFLP technology (Figure 1) (Helentjaris et al., 1985). RFLPs, at least for many cultivated plant species, raised discriminational ability to a level where the term "fingerprinting" became appropriate coinage. RFLPs can allow a sufficiently dense and informative sampling of genomic diversity that genetic distances, meaningfully reflective of that diversity, can be calculated. For example, RFLPs can assign inbred lines into heterotic groups and further into subdivisions of these groups (Ajmone-Marsan et al., 1992a,b; Lee et al., 1989; Livini et al., 1992; Melchinger et al., 1991; 1992; Messmer et al., 1991; 1992a,b;). For

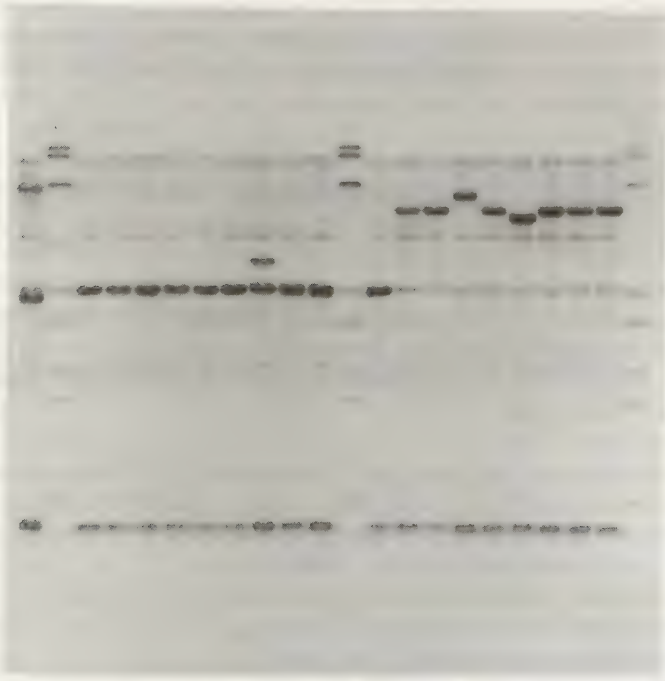


Figure 1. RFLP profiles of 19 maize inbred lines. Molecular weight standard "ladders" were loaded into (from left to right) lanes 2, 12 and 22.

inbreds connected by pedigree, RFLPs show distances that correlate highly with distances calculated from other multigenic data such as pedigree, F1 yield and heterosis (Smith et al., 1990). Lastly, RFLPs allow the construction of detailed genetic maps. These maps include the approximate locations of agronomically important regions (Quantitative Trait Loci or QTLs). The limited quality and genomic coverage afforded by isozymes effectively prohibited such effective use of these data in applied breeding programs. In contrast, RFLPs offer a wealth of diversity among elite germplasm to a level of abundance that has arrested the attention of plant breeders and associated researchers. Similarity of RFLP profiles among F1 maize hybrids can indicate similar parentage (Smith and Smith, 1991).

The Impetus to Develop DNA Amplification Methods

Research with the numerous samples engendered by inclusion of many individuals from each of several segregating populations, rapidly and glaringly revealed the practical limitations of RFLPs. The technology is not at all well adapted to high volume sample throughput in short-time periods. The potential impact of DNA amplification technologies such as the Polymerase Chain Reaction (PCR) or thermocycling, therefore, has become especially attractive.

DNA amplification can offer advantages of increased sample throughput efficiency by 1) the use of small input levels of DNA; 2) the avoidance of radiation in detection; 3) the potential avoidance of gel based analyses; and 4) the avoidance of Southern transfers. For support of plant breeders, PCR systems have allowed implementation of laboratory

robotics and rapid assays run in parallel. Rapid +/- detection systems could be wholly integrated with computer based sample tracking, data acquisition, analysis and reporting. Potentially, at least some assays could be envisioned as being transferable directly to the field in the form of kits.

INTERPRETATION OF MOLECULAR MARKER DATA

A very strong impetus for the development of new molecular marker technologies arises from the need of laboratories to match the exacting demands and aspirations of plant breeders. Therefore, it is critical to recognize that criteria for the effective use of markers in variety identification differ from those required for direct support to breeders. In the latter case (breeders' support), the necessities of high volume throughput, very low sample cost and very rapid analysis time reign supreme.

In contrast, for effective use of molecular markers in varietal identification the following criteria are important:

a. discriminational ability;
b. degree of freedom from Genotype x Environmental (G x E) interaction;
c. high level of standards and protocols to assure the generation of quality data;
d. objectivity of scoring and of databasing varietal profiles;
e. use of peer reviewed and published methodologies;
f. well understood genetic control;
g. applicability to determination of pedigree;
h. applicability to determination of EDV status;
i. publicly available database of profiles; and
j. cost effective and reasonably timely data generation.

For purposes of varietal identification, sample numbers will almost always be much reduced in comparison to the number of individuals that must be profiled for just one or a very few segregating populations. More time will usually be available for varietal profiling in comparison to the rigorous time demands that are everyday components of plant breeding. Plus/minus molecular marker assays carry a premium in meeting the demands made by breeders. In contrast, for varietal identification purposes, unless comparison to one specific variety is the sole object, such allelic +/- assays would be inefficient. In varietal identification profiling, a system based on +/- assays would require an individual assay for each allele of each molecular marker target site (or locus). Negative results could be due either to absence of the allele or failure of the assay. Also, other alleles could be present but would remain undetected without the provision of an allele specific assay. Therefore, at least until sequence data can be available at less cost and most, if not all, alleles can be more effectively and reliably assayed, then electrophoretic separation systems will remain the preferred means by which variants are separated and become discretely scored for varietal identification purposes. Scientifically sound procedures must be in place to allow repeatably reliable and objective means of translating quantitative electrophoretic band mobility data into qualitative variant or allele scores (Brumback et al., 1992; Galbraith et al., 1991; Gill and Werrett, 1990).

THE USE OF DNA AMPLIFICATION TECHNOLOGIES IN VARIETAL IDENTIFICATION

Currently available PCR based methods that are being applied to varietal identification may be classified among three groups depending upon the degree to which the primer oligomer sequence is knowingly homologous to the amplification target

sequence region of the cultivar.

Non-random and Semi-random Primer Systems

Primers that are non-random or semi-random with respect to target DNA can only be constructed when DNA sequence data within the target varieties are known. Such data are available from GenBank for specific species and for individually known genes or sequences such as intron/exon splice junctions. Where sequence data are conserved across species, then the primers that are manufactured can have applicability across these species. Unfortunately, most sequence data are not currently available. Therefore, simple repeat regions that will reveal polymorphism across varieties must be identified and then sequence data of flanking regions must be obtained. While becoming technically more feasible, this exercise remains demanding of resources. Amplification of DNA using non-random primers allows annealment of primer and target DNA to occur only at a high stringency. Therefore, clear and repeatable amplification products would be expected.

Random Primer Systems

Random primer systems avoid the initial cost of finding and sequencing relevant regions of the genome. With random primer amplification, the degree of homology between target DNA and primer DNA can range from various unknown degrees of incompleteness to complete homology. As the degree of homology declines, interactions from other components of the reaction mixture could begin to significantly influence the amplification process. Hence, opportunities could occur to affect the consequent profiles. Rigorous experimentation is required with random primers to allow the development of procedures that can generate reliably repeatable profiles (Devos and Gale, 1992). Nevertheless, acceptable procedures have been developed and demonstrated for many diverse species (Skroch et al., 1992; Smith and Chin, 1992; Thormann and Osborn, 1992; Weeden et al., 1992).

Random primer mediated methods are particularly appealing since they can generate equivalently numerous polymorphisms with less effort and resources when compared either to RFLP technology or to non-random primer methods. Random primer methods have been a great boon in studies of genetic variability, for example, in species with long life cycles such as forest trees (Grattapaglia et al., 1992). They have also been useful in situations where the level of economic importance of the crop had hitherto rendered more expensive RFLP analyses an unwarranted proposition. However, large and long-term varietal profiling ventures with annual additions to the database would likely be advantaged in utilizing technologies with a higher level of long-term repeatability where that is feasible.

Specific Primer Based Technologies

Random Primers

Randomly Amplified Polymorphic DNA (RAPDs) (Figure 2) (Williams et al., 1990) and Arbitrarily Primed - Polymerase Chain Reaction (AP-PCR) (Figure 3) (Welsh and McClelland, 1990; Welsh et al., 1991) constitute the first two random primer mediated profiling technologies that reached public notice. Both methods utilize 10 base oligomer primers. Amplification products are generally separated in agarose gels and are revealed following staining with Ethidium Bromide. (However, initial AP-PCR was performed using acrylamide gels and radiolabelled amplification products). Using either technique, profiles of individual varieties are almost always multibanded. Individual

Figure 2. RAPD profiles of nine inbred lines of maize generated by a single 10 mer primer. Amplifications of each inbred were conducted in duplicate and loaded adjacently into the gel. The right-most lane contains a molecular weight standard. The adjacent blank lane was loaded with the minus maize DNA control amplification reaction.

bands are of different intensities because of their differential amplification amounts. One or other of these technologies have been successfully applied to varietal discrimination among a very wide variety of cultivated plant species.

RAPDs do not appear to provide greater additional power of discrimination for species where a high ability has already been shown by RFLPs. For example, using RAPDs we did not achieve a greater power of discrimination among related inbred lines of maize than had already been shown by RFLPs (Smith and Chin, unpubl.). However, within species where either RFLPs or conventional RAPD techniques have shown relatively little discriminatory ability, the separation of RAPD amplification products in denaturing gradient gel electrophoresis (DGGE) has provided an enhanced power of identification (Dweikat et al., 1993; He et al., 1992). While individual RAPD or AP-PCR primers may not always provide an enhanced power of discrimination, they do constitute a means of unparalleled speed and efficiency to reveal many differences between specific inbred lines and varieties. For example, these methods can rapidly reveal polymorphisms between genotypes using bulk segregant analysis (Giovannoni et al., 1991). Similarly, they can rapidly generate markers for introgressed segments in Near Isozymic Lines (NILs), (Michelmore et al., 1991). It is also possible to more rapidly screen tens or hundreds of primers for their discriminatory ability than it is an equal number of RFLPs. Therefore, these methods can rapidly generate a list of polymorphisms between most, if not all, inbred lines or cultivars even if they are very closely related by pedigree. However, it must be cautioned that these data alone can

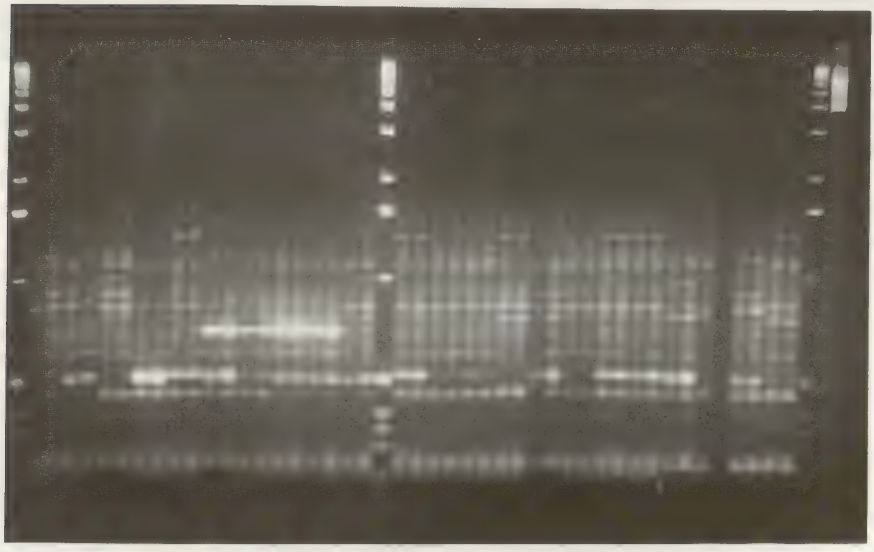


Figure 3. Forty-four maize inbred samples amplified with 10 mer by AP-PCR and run out in an agarose gel. Lanes (from left to right) 1, 2, 23, 24, 49 and 50 were run with molecular weight standards.

provide no real evidence of discriminatory power or of overall genetic similarity among the specific germplasm under analysis. Overall discriminatory power can only be established once a range of genotypes of known pedigree, and which encompasses a breadth of genetic diversity, has been profiled using genetic markers that provide a reasonably comprehensive survey of the genome.

No profiles can be properly scored unless account is taken of individual band repeatability. Such care is particularly important with random primer directed amplification since the technology may be especially prone to interaction during annealment and subsequent extension, producing an effect upon amplification products. Special care and concern would likely accompany the scoring of faint to moderately intense bands. Profiles should be generated and scored in replicate. Use of negative controls is an imperative. A degree of objectivity in scoring should be implemented using molecular weight and genomic standards. Band scoring using either genomic standards and/or some objectively determined threshold of band intensity is mandatory.

It may be difficult, if not impossible, to obtain a comprehensive knowledge of the genetic control of random primer mediated polymorphisms. For example, Johns (1992) found that amplification products of apparently similar lengths generated by the same primers but from different target genotypes frequently mapped to different locations in the maize genome. An incomplete knowledge of genetic control undermines abilities to determine genetic distance and to validate pedigree. Therefore, special care and attention will be necessary for application of random primer mediated profiles to the determination of EDV status.

DNA Amplification Fingerprinting (DAF), (Caetano-Annolles et al., 1991) is a

method closely similar in its process to RAPD and AP-PCR. With DAF shorter 5-7 mer primers are used. Amplification products are separated in acrylamide gels and silver stains are used. DAF has been used across a wide spectrum of genera, soybean being the predominant cultivated plant species within which the technique has been applied. Since the underlying technologies of these random primer methods are so congruent, similar experiences and conclusions will likely apply to DAF as have already been expressed for RAPD and AP-PCR technologies.

Amplification Fragment Length Polymorphisms (AFLPs) (Figure 4) is a related but significantly different technology to those previously described. AFLPs do not require advanced knowledge of target DNA sequence. However, the nature of their invention

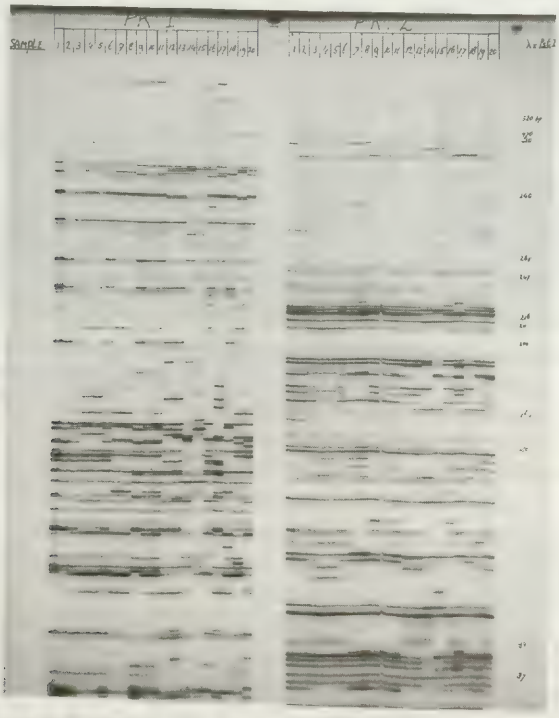


Figure 4. AFLP profiles of maize inbreds and hybrids. Profiles generated by each of two primers are presented. Center and right lanes are molecular weight standards.

allows primer annealment and extension to occur at relatively high levels of stringency. Therefore, AFLP technology offers the promise of providing profiles that are of an enhanced repeatability and that have few bands of moderate to low relative intensities. AFLP technology is expected to be fully reported upon in the scientific literature very soon. It has been developed by Keygene (Marc Zabeau, pers. comm.). Preliminary data show AFLPs to have an equivalent discriminatory power to RFLPs, RAPDs, or AP-PCR. The technology may be more efficient to implement in varietal identification if results from a small number of primers (e.g., 10-15) are equivalent to those generated by a greater number (e.g., 100) of single copy RFLP probes. The multibanded nature of the AFLP profile can be informationally rich provided means can be implemented to allow reliable scoring. It will also be important to obtain genetic control data that are valid across an appropriate range of germplasm.

Semi-random Primer Methods

Weining and Langridge (1991) used PCR with pairs of primers where one primer member was targeted to known sequence of an intron splice junction and the other was generated at random. Using this procedure, one pair of primers allowed 21 barley varieties to be separated into 15 groups; use of additional primer pairs provided unique identification of all the varieties. These methods could have application in other species. Consistent evaluatory criteria (mentioned previously) would need to be applied in order to reach a conclusion as to if, and how, this method would be used in varietal application.

Non-random Primer Methods

Amplification of products between known sequence sites will reveal polymorphisms based upon either 1) a difference in sequence length between primer annealing sites; or 2) a difference in sequence between sites which can be revealed through restriction enzyme digest patterns; or 3) a difference in sequence at one or both annealing sites. Amplification target sites can include coding regions of genes [e.g., gamma-gliadin wheat proteins (D'Ovidio et al., 1990; 1992); amylase barley protein (Weining and Langridge, 1991)]; or non-coding regions. Non-coding regions targeted by DNA amplification have either been sites of demonstrated discriminational ability and known genetic map location such as single-copy RFLPs (Williams et al., 1991; Livneh et al., 1992) or regions that promise to be hypervariable since they encompass short sequences arrayed in tandem repeats (satellites) (Akkaya et al., 1992; Phillips et al., 1992; Wu and Tanksley, 1992; Zhao and Kochert, 1992; Ziegle et al., 1992). DNA amplification of RFLPs provides a means to establish a +/- assay and may, therefore, be especially appropriate for providing molecular marker support to plant breeding rather than for implementation in varietal identification.

Amplification of mini- or micro-satellite regions has usually resulted in length polymorphisms that are revealed following electrophoresis. Use of different fluorescent tags for the products of each of several amplification target locations provides increased efficiency through the ability to multiplex separations. Enhanced reliability and precision in data scoring can be provided through the additional multiplexing of control DNA molecular weight standards (Mayrand et al., 1992). Simpler procedures are becoming available to locate satellite regions and hence to generate sequence data for conserved flanking regions that will provide the anchor points for primer annealment. Considerable sequence data are already public knowledge and are available in GenBank. Initial data suggest that microsatellites may allow an enhanced ability to discriminate among varieties of cultivated species such as wheat and tomato that were hitherto recalcitrant to allowing many polymorphisms to be revealed among elite cultivars. The data are also revealed as codominant variants; genetic control information may be quite readily obtainable and applicable across a range of germplasm.

FURTHER TECHNOLOGIES AND CONCLUSIONS

Variations upon existing methodologies and more extensive changes with consequent new possibilities for their integration into the development of fresh approaches to the revelation of genetic diversity occur at an astonishingly rapid rate. Research results from the fields of human , small mammal, fungal and viral genetics and molecular biology are particularly forthcoming. There will not likely be any shortage of methods by which polymorphisms can then also be revealed among cultivated plant varieties. The challenge will continue to be to employ appropriate methods in a fashion

suitable to the demands made by the purposes of varietal identification. Therefore, plant breeding, agriculture and society as a whole can be well served provided we are successful in defining and accomplishing the necessary tasks.

REFERENCES

Ajmone-Marsan, P., Livini, C., Messmer, M.M., Melchinger, A.E., Franceschini, P., Monfiedini, G. and Motto, M. 1992a. Comparison between cluster analyses from RFLP and pedigree data of inbred lines related to Stiff Stalk heterotic group. Maize Genet. Coop. Newslett. 66:18-20.

Ajmone-Marsan, P., Livini, C., Messmer, M.M., Melchinger, A.E. and Motto, M. 1992b. Cluster analysis of RFLP data from related maize inbred lines of the BSSS and SC heterotic groups and comparison with pedigree data. Euphytica 60:139-148.

Akkaya, M.S., Bhagwat, A.A. and Cregan, P.B. 1992. Length polymorphisms of simple sequence repeat DNA in soybean. Genetics 132:1131-1139.

Brumback, T.B., Smith J.S.C., Smith, O.S. Tenborg, R., Wall, S.J., Beghtol, T. and Hartman, M.R. 1992. Variability of molecular weight determination of RFLP bands for genetic fingerprinting in maize. Agron. Abstr. p. 188.

Caetano-Anolles, G., Bassam, B.J. and Gresshoff, P.M. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. CSSA Meetings, Minneapolis, ASA, Madison, WI. Bio/Technol. 9:553-557.

Devos, K.M. and Gale, M.D. 1992. The use of random amplified polymorphic DNA markers in wheat. Theor. Appl. Genet. 84:567-572.

D'Ovidio, R., Tanzarella, O.A. and Porceddu, E. 1990. Rapid and efficient detection of genetic polymorphisms in wheat through amplification by polymerase chain reaction. Plant Mol. Biol. 15:169-171.

D'Ovidio, R., Tanzarella, O.A., Masci, S., LaFiandra, D. and Porceddu, E. 1992. RFLP and PCR analyses at Gli-1, Gli-2, Glu-1 and Glu-3 loci in cultivated and wild wheats. Hereditas 116:79-85.

Dweikat, I., Mackenzie, S., Levy, M. and Ohm, H. 1993. Pedigree assessment using a RAPD-DGGE in cereal crop species. Theor. Appl. Genet. 85:497-505.

Galbraith. D.A., Boag, P.T., Gibbs, H.L. and White, B.N. 1991. Sizing bands on autoradiograms: A study of precision for scoring DNA fingerprints. Electrophoresis 12:210-220.

Gill, P. and Werrett, D.J. 1990. Interpretation of DNA profiles using a computerized database. Electrophoresis 11:444-448.

Giovannoni, J.J., Wing, R.A., Ganal, M.W. and Tanksley, S.D. 1991. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping populations. Nucleic Acids Res. 19:6553- 6558.

Grattapaglia, D., Chaparro, J., Wilcox, P., McCord, S., Werner, D., Amerson, H., McKeand, S., Bridgewater, F., Whetten, R., O'Malley, D. and Sederoff, R. 1992. Mapping in woody plants with RAPD markers: Application to breeding in forestry and horticulture, *in:* "Symposium: Applications of RAPD Technology to Plant Breeding," 1 Nov., 1992, Minneapolis, ASA, Madison, WI.

He, S., Ohm, H. and Mackenzie, S. 1992. Detection of DNA sequence polymorphisms among wheat varieties. Theor. Appl. Genet. 84:573-578.

Helentjaris, T., King, G., Slocum, M., Siedenstrong, C. and Wegman, S. 1985. Restriction fragment length polymorphisms as probes for plant diversity and their development as tools for applied plant breeding. Plant Mol. Biol. 5:109-118.

Johns, M.A. 1992. Comparison of RAPD maps for two sets of maize recombinant inbred lines, Abstr. p. 32, *in:* Poster Sessions, Plant Genome I Meeting, Nov. 9-11, San Diego,CA, Scherago Intl.

Lee, M., Godshalk, E.B., Lamkey, K.R. and Woodman, W.L. 1989. Association of restriction fragment length polymorphisms among maize inbreds with agronomic performance of their crosses. Crop Sci. 29:1067-1071.

Livneh, O., Vardi, E., Stram, Y., Edelbaum, O. and Sela, I. 1992. The conversion of a RFLP assay into PCR for the determination of purity in a high pepper cultivar. Euphytica 62:97-102.

Livini, C., Ajmore-Marsan, P., Melchinger, A.E., Messmer, M.M. and Motto, M. 1992. Genetic diversity of maize inbred lines within and among heterotic groups revealed by RFLPs. Theor. Appl. Genet. 84:17-25.

Mayrand, P.E., Corcoran, K.P., Ziegle, J.S., Robertson, J.M., Hoff, L.B. and Kronick, M.N. 1992. The use of fluorescence detection and internal lane standards to size PCR products automatically. Appl. and Theoret. Elect. 3:1-11.

Melchinger, A.E., Boppenmaier, J., Dhillon, B.S., Pollmer, W.G. and Herrmann, R.G. 1992. Genetic diversity for RFLPs in European maize inbreds: II. Relation to performance of hybrids within versus between heterotic groups for forage traits. Theor. Appl. Genet. 84:672- 681.

Melchinger, A.E., Messmer, M.M., Lee, M., Woodman, W.L. and Lamkey, K.R. 1991. Diversity and relationships among U.S. maize inbred lines revealed by Restriction Fragment Length Polymorphisms. Crop Sci. 31:669-678.

Messmer, M.M., Melchinger, A.E., Boppenmaier, J., Brunklaus- Jung, E. and Herrmann, R.G. 1992a. Relationships among early European maize inbreds. I. Genetic diversity among flint and dent lines revealed by RFLPs. Crop Sci. 32:1301-1309.

Messmer, M.M., Melchinger, A.E., Boppenmaier, J., Herrmann, R.G. and Brunklaus-Jung, E. 1992b. RFLP analysis of early maturing European maize germplasm. Theor. Appl. Genet. 83:1003-1012.

Messmer, M.M., Melchinger, A.E., Lee, M., Woodman, W.L., Lee, E.A. and Lamkey, K.R. 1991. Genetic diversity among progenitors and elite lines from the Iowa Stiff Stalk Synthetic (BSSS) maize population: comparison of allozyme and RFLP data. Theor. Appl. Genet. 83:97-107.

Michelmore, R.W., Paron, I. and Kesseli, R.V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88:9828-9832.

Phillips, W., Chapman, C. and Jack, P. 1992. Identification and characterization of hypervariable simple sequence DNA elements in tomato (*Lycopersicon esculentum)*, Abstr. p. 30, *in*: Poster Sessions, Plant Genome I Meeting, Nov. 9-11, San Diego, CA. Scherago Intl.

Skroch, P., Tivang, J. and Nienhuis, J. 1992. Analysis of genetic relationships using RAPD marker data, *in*: "Symposium, Applications of RAPD Technology to Plant Breeding," 1 Nov., 1992, Minneapolis, ASA, Madison, WI.

Smith, J.S.C. and Chin, E. 1992. The utility of random primer-mediated profiles, RFLPs, and other technologies to provide useful data for varietal protection, *in*: "Symposium: Applications of RAPD Technology to Plant Breeding," 1 Nov., 1992, Minneapolis, ASA, Madison, WI.

Smith, J.S.C. and Smith, O.S. 1991. Restriction fragment length polymorphisms can differentiate among U.S. maize hybrids. Crop Sci. 31:893-899.

Smith, O.S., Smith, J.S.C., Bowen, S.L., Tenborg, R.A. and Wall, S.J. 1990. Similarities among a group of elite maize inbreds as measured by pedigree, F1 grain yield, grain yield heterosis and RFLPs. Theor. Appl. Genet. 80:833-840.

Thormann, C.E. and Osborn, T.L. 1992. Use of RAPD and RFLP markers for germplasm evaluation, *in*: "Symposium, Applications of RAPD Technology to Plant Breeding," 1 Nov., 1992, Minneapolis, ASA, Madison, WI.

Weedon, N.F., Timmerman, G.M., Hemmat, M., Kneen, B.E. and Lodhi, M.A. 1992. Inheritance and reliability of RAPD markers, *in*: "Symposium: Applications of RAPD Technology to Plant Breeding," 1 Nov., 1992, Minneapolis, Crop Sci. Soc. Amer.

Weining, S. and Langridge, P. 1991. Identification and mapping of polymorphisms in cereals based on the polymerase chain reaction. Theor. Appl. Genet. 83:209-216.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids. Res. 18:7213-7218.

Welsh, J., Honeycutt, R.J., McClelland, M. and Sobral, B.W.S. 1991. Parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (AP-PCR). Theor. Appl. Genet. 82:473-476.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990 DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Williams, M.N.V., Pande, N., Nair, S., Mohan, M. and Bennett, J. 1991. Restriction fragment length polymorphism analysis of polymerase chain reaction products amplified from mapped loci of rice (*Oryza sativa* L.) genomic DNA. Theor. Appl. Genet. 82:489-498.

Wu, K. and Tanksley, S.D. 1992. Abundancies, polymorphisms and genetic mapping of microsatellites in rice, p. 55, *in*: Abstr. Poster Sessions, Plant Genome I Meeting, Nov. 9-11, San Diego, CA, Scherago Intl.

Zhao, S. and Kochert, G. 1992. Characterization and genetic mapping of a short, highly repeated, interspersed DNA sequence from rice (*Oryza sativa* L.). Mol. Gen. Genet. 231:353-359.

Ziegle, J.S., Su, U., Corcoran, K.P., Nie, L., Mayrand, .E., Hoff, L.B., McBride, L.J., Kronick, M.N. and Diehl, S.R. 1992. Application of automated microsatellite loci. Genomics 14:1026-1031.

POLYMERASE CHAIN REACTION WITH UNIVERSAL PRIMERS (UP PCR) AND ITS APPLICATIONS TO PLANT GENOME ANALYSIS

S.A. Bulat[1]*, N.V. Mironenko[2], M.N. Lapteva[3] and P.P. Strelchenko[3,1]
[1]St.Petersburg Nuclear Physics Institute, Gatchina, 188350, Russia
[2]All Russian Plant Protection Institute, St.Petersburg, 189620, Russia
[3]N.I. Vavilov Institute of Plant Industry (VIR), St.Petersburg, 190000, Russia

SUMMARY

A new method of genotyping, species identification and estimation of genetic relationships of organisms is presented. It is based on PCR using a single universal (random) primer - Universally Primed PCR (UP PCR, as analog of RAPD PCR). For the first time, the property of UP PCR amplified DNA to hybridize only with the genetically related organisms is described. The amplified DNA was also shown to be primer specific in DNA hybridization. The usefulness of UP PCR technique to analyze genotypes and the genetic relationships (pedigree) of varieties, species and intergeneric hybrids in potatoes and wheat is demonstrated. This technique also allows one to identify the varieties and species and verify their seed purity for the purposes of taxonomy, bioconservation and breeding.

INTRODUCTION

The problem of conservation of plant diversity is very real and important for Russia and other republics of the former USSR. Thus, the previous DNA Bank Net Meeting, held in London in 1991, evoked great interest within the scientific community in these countries. That is why we developed the project for the conservation of plant genes. Some research organizations in Russia have joined this project. One of the first steps in this project is a mastering of methods for DNA isolation and conservation as well as methods of registration and systematization of world plant genetic resources including the use of molecular markers. Earlier we reported that in our work we used RFLP markers (Strelchenko et al., 1992).

At present in molecular genetics, in addition to RFLPs, PCR markers are gaining more widespread utilization. These markers are the DNA fragments, amplified in the polymerase chain reaction (PCR). Polymorphisms of these fragments is the basis for locus and genome mapping. Several PCR methods suitable for organism genotyping are UP PCR (Bulat and Mironenko, 1990; 1992; Bulat et al., 1992a), RAPD PCR (Williams et al., 1990) and AP PCR (Welsh and McClelland, 1990). RAPD markers are of more widespread use because of the inexpensive nature of 10 mer primers of random nucleotide sequence and simplicity of analysis (agarose gel electrophoresis). They are being already used for varieties, subspecies and species genotyping of plants, as it has been demonstrated in soybean, maize, alfalfa, peanut, rye and other crops (Echt et al., 1992; Halward et al., 1991; Hedrick, 1992; Heusden and Bachmann, 1992; Lanham et al., 1992; Rogowsky et al., 1992; Welsh and McClelland, 1990; Williams et al., 1990) as well as for marking of genomes. The first such genetic linkage map was already constructed for *Arabidopsis thaliana* (Reiter et al., 1992).

*To whom correspondence should be addressed.

As RAPD techniques were developed, the main effort was made on identification of loci (Williams et al., 1990) with the aim of using RAPDs for mapping. In this connection amplified products representing DNA repeats, as a rule, are not analyzed. RAPD-like techniques, which we offer [patent No. 4577254/13 (137743)(Russia), priority from 20.11.89] are also suitable for loci identification, but the emphasis has been placed on the production of more informative, complicated PCR patterns of amplified DNA. This DNA may consist both of DNA repeats and unique loci. Such techniques satisfy most of the purposes of genotyping, identification of species, subspecies, varieties, strains and serotypes of various organisms as well as investigations of their relationships. We demonstrated this with fungi, bacteria (Bulat and Mironenko, 1990; 1992; Bulat et al., 1991; 1992a,b), protozoa, plants and mammalia (unpublished data).

UP PCR TECHNIQUE

UP PCR technique consists of DNA amplification of any organism with single universal primers, which are the kind of primers with random nucleotide sequences. As a result, one can produce a complicated, amplified DNA pattern (sizes) which are specific not only for the certain genome, but also, frequently, for this species (strains, serotypes) of organisms. In addition, homology of fragments of amplified DNA is revealed only in genetically related organisms. This is true only for DNA which has been amplified with the same primer, i.e., amplified DNA is primer specific in DNA hybridization (Bulat and Mironenko, 1992; Bulat et al., 1992a; see text under Figure 5).

For complicated genomes, PCR products may represent both unique and repetitive DNA fragments, which as a rule do not contain open reading frames. In phage and plasmid DNA models, it has been shown that random DNA sequences flanked by inverted microrepeats (present in any complex genome) are the sites of prime ring by universal oligonucleotides. In experiments with the mixture of heterogeneous DNAs, it was shown that UP PCR oligonucleotides (8 - 20 nucleotides long, annealed at 55°C) primered the genome DNA in maximum possible homology (in sites with maximum binding constants). A change of any base of oligonucleotide leads to different DNA prime ring sites according to new binding constants and, as a result, the pattern of amplified DNA is changed.

The effect of homology of amplified DNA with random primers in the group of related organisms allowed us to examine the nature of interspecific differences of DNA level in another way. These DNA differences we suggest are connected with certain threshold values of nucleotide substitutions in uncoding DNA regions, for which a higher degree of divergence is characteristic, in comparison with coding regions (Nei, 1987).

As mentioned above, universal primers with high specificity are connected with certain sites in the genome. If at such sites nucleotide substitutions arise, the pattern and homology of amplified DNA will be changed. As a result of this, such a genome will differ from the initial one. For example, for higher organisms (primates) for whom nucleotide substitution rate is lower (Britten, 1986; Li et al., 1987; Seino et al., 1992), the homology of amplified DNA may likely be on the level of the higher taxon (genus). For a heterogeneous group of organisms, such as plants, the interpretation of species differences using amplified DNA may not be synonymous because of various sizes of their genomes, parts of repetitive DNA (Bennett and Smith, 1991; Lapitan, 1992) and, therefore, various mutation rates (Drake, 1992), diversity of their life cycles, ways of propagation and peculiarities of generation and maintenance of neutral mutations (Nickrent and Franchina, 1990; Shattuck-Eidens et al., 1990). Nevertheless, the

property of homology of amplified DNA provides information about the genetic relationship of analyzed organisms.

Using the plants of genera *Solanum* and *Triticum* demonstrates the suitability of UP PCR techniques both for marking (genotyping) of varieties and species and for characteristics of their genomes in terms of their genetic relationships.

MATERIALS AND METHODS

Plant materials and sources are presented in Tables 1 and 2. Plant cells were lysed by mixing with 200 ml of lysis buffer (50 mM TRIS-HCl, pH 7.8, 50 mM EDTA, 100 mM NaCl, 2% N-Lauroyl Sarcosine, 500 mM 2-Mercaptoethanol, 1000 g/ml Proteinase K) followed by grinding in liquid nitrogen and heated at 65°C for 1 hr. Then NaCl was adjusted to 1 M and an equal volume of chloroform/octanol mixture (24:1) was added to denature proteins. After centrifugation at 12,000 g for 2 min, the upper, clear, aqueous phase was withdrawn, and the DNA was precipitated by adding 0.6 volume of isopropyl alcohol. The pellet was rinsed once with 70% ethanol and redissolved in TE buffer (1 mM TRIS/HCl, 0.1 mM EDTA). 0.1 ml of the DNA solution was used for PCR.

PCR (30 cycles) was performed according to Saiki et al. (1988) using 2-3 units of thermostable DNA polymerase (O.K. Kaboev, Petersburg Nuclear Physics Institute, Russia) in the presence of 4mM $MgCl_2$, 0.6 mM each of dNTPs and 40 ng of primers. Single universal primers (16-17 mers) of our design and production were used. (Code: Sequence): **45**:GGA TCC AAA ACG ACG GCC AGT; **3-2**:TAA GGG CGG TGC CAG T; **0.3.1**:CGA GAA CGA CGG TTC T; **AS15**: Patent Pending.

GENOTYPING AND ANALYSIS OF GENETIC RELATIONSHIP OF VARIETIES AND SPECIES OF POTATOES

Identification of potato varieties is very important both for protection of a breeder's rights and for verification of seed purity and stability. At present genotyping and varietal identification on polymorphic proteins are widely adopted. More than 600 potato varieties are registered in Europe according to their agronomic traits, protein biotypes and isozymes. Recently it was shown that for varietal identification, RFLP markers are more suitable. RFLPs have a number of advantages over protein markers (Gebhardt et al., 1989a). For identification of many potato varieties, it appeared adequate to use a single DNA probe and single restriction enzyme (Gorg et al., 1992). The use of such a technology based on PCR is just being planned (Gebhardt et al., 1989b).

In our investigations we used tubers of both cultivated and old potato varieties of various selected groups (Table 1).

From a number of universal primers, 0.3-1 primer was chosen, which in UP PCR was good for distinguishing all the six varieties (Figure 1). Each variety has a specific invariable PCR pattern. At the same time, separate fragments of the potato patterns occur in different combinations that indicate relatedness in the varietal genomes. This relationship is possibly caused by the use of certain varieties as common "parents" (wild species). Having a number of various varieties and wild species, one can determine the relationships of a certain variety and evaluate the contribution of certain parents in the varietal genome.

Table 1. List of potato wild species and varieties used in these analyses. Series are cited according to Hawkes (1979).

Series	Species (N)*	VIR catalogue number
Demissa	*Solanum demissum* (9)	K 2076
Longipedicellata	*S. stoloniferum* (6)	K 18927
Commersoniana	*S. chacoense* (3)	K 19991
Tuberosa	*S. tuberosum* subsp. *andigena*	
	var. *mammiformae* (3)	K 15415
	var. *imilla negra* (3)	K 4716
	S. phureja (7)	K 18156
	S. verrucosum (12)	K 3537
	S. sparsipilum (9)	K 9808
	S. vernei (6)	K 11433
	S. spegazzinii (1)	K 11428
	S. microdontum (7)	K 12576
	S. tuberosum varieties:	
	Detskoselsky (3)	
	Nevsky (3)	
	Izora (23)	
	Sante (23)	
	Laimdoty (3)	
	Pribrezhny (3)	

(N)* = number of plants analyzed.

Primers synthesized based on inverted repeats of a potato patatine gene (Pikaard et al., 1986) were tested for their ability to determine varietal and species differences. No principal differences between them, and random primers were observed (data not shown). However, PCR varietal patterns, obtained with the use of genome specific primers, were complex in terms of their interpretation of genome specific fragments identification. That is why the preference was given to universal (random) primers.

Shoots of tubers of Izora and Sante varieties (taken as a random number of 20 tubers) were tested to determine the stability of UP PCR patterns. Both varieties were genetically homogeneous and no differences between individuals were seen in any fragments.

To obtain the information about the genome structure of *S. tuberosum* which is of hybrid origin and for which not all "parents" are known (Hawkes, 1979; Grun, 1990), we analyzed a number of potato species by the UP PCR method with primer 0.3-1 (Figure 2). These consisted of both hypothetical wild ancestors as well as potato breeding species most utilized worldwide as donors of agronomic traits (Ross, 1986). Some species were represented by several plants (Table 1). In addition, several species (e.g., *S. sparsipilum, S. vernei* and some others) and their UP PCR biotypes were used (Figure 2).

As seen in Figure 2, 0.3-1 primer differentiates all the potato varieties and species used in the analysis . It is likely that the similarity of patterns reflects genetic relationship of species. For example, wild species from *Tuberosa* series *S. sparsipilum, S. spegazzinii, S. vernei* and *S. microdontum* are characterized by similar patterns as well as cultivated species and varieties of *S. tuberosum* subsp. *andigena, S. phureja* and *S. tuberosum* (varieties).

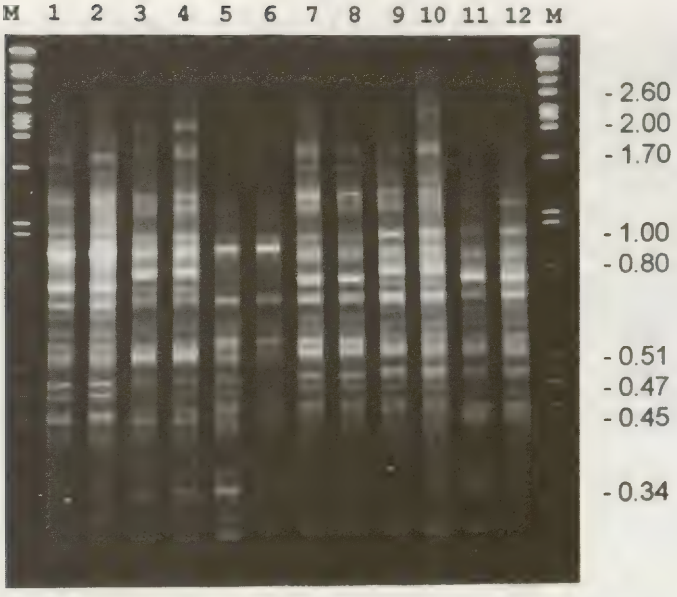


Figure 1. Genotyping of potato varieties by UP PCR technique with the primer 0.3-1. Lane M = Pst I-fragments of lambda DNA (fragment size are shown in kb). Lanes 1,2 - Pribrezhny; 3,4 - Laimdoty; 5,6 - Sante; 7,8 - Izora; 9,10 - Nevsky; 11,12 - Detskoselsky. For each variety 2 plants were analyzed. Electrophoresis in 5.5% PAAG in TBE buffer.

Moreover, taking into account the complexity of the visual interpretation of UP PCR patterns by size fragments and also the homology of amplified DNA in related genomes, we conducted an analysis for DNA homology of PCR products. Results of blot hybridization with species and varieties (Figure 2) using labeled amplified DNA of the Sante variety are shown in Figure 3A. Along with *S. tuberosum* varieties, hybridization was also obtained with other species. In our opinion, this may indicate that these species or their relatives have taken part in formation of *S. tuberosum* genome (Ross, 1986). Very similar hybridizational patterns were obtained with *S. tuberosum* ssp. *andigena* and *S. phureja*. This is not surprising because they are closely related species (Grun, 1990). The representatives of other series (*S. demissium* and *S. stoloniferum*) also gave hybridizations of fragments and this confirms their considerable contribution to the *S. tuberosum* genome (Ross, 1986). The weakest signals were obtained from *S. vernei* although this species was used in the development of the Sante variety.

Rehybridization of this blot with labeled, amplified DNA of *S. vernei* showed a closely related group of wild species from Tuberosa series (data not shown). This group also had similar UP PCR patterns (Figure 2). *Solanum tuberosum* varieties showed considerable polymorphism with this probe.

Hybridization of amplified UP DNA may be also carried out with the use of separate fragments from DNA patterns. For example, a fragment which was eluted from

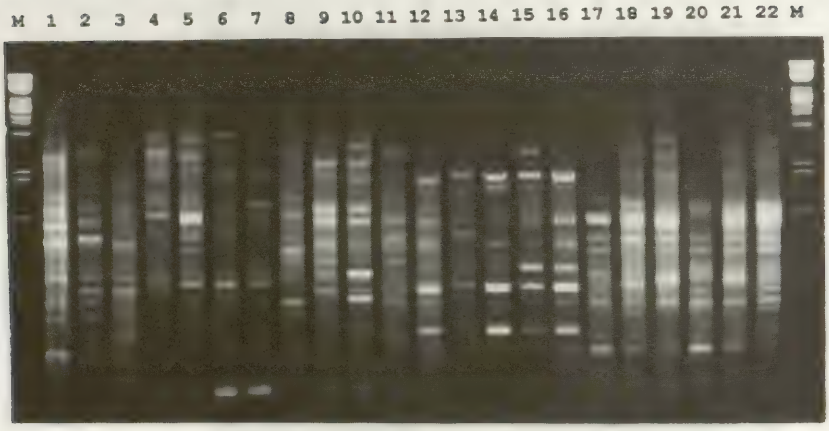


Figure 2. Genotyping of *Solanum* species by UP PCR technique with the primer 0.3-1. Lanes 1,2 - *S. demissium*; 3 - *S. stoloniferum*; 4 - *S. tuberosum* ssp. *andigena* var. *mammiformae*; 5 - *S. tuberosum* ssp. *andigena* var. *imilla negra*; 6,7 - *S. phureja*; 8 - *S. chacoense;* 9,10 - *S. verrucosum*; 11,12 - *S. sparsipilum;* 13,14 - *S. vernei;* 15 - *S. spegazzinii;* 16 - *S. microdontum.* Varieties of *S. tuberosum*: 17 - Detskoselsky, 18 - Nevsky, 19 - Izora 20 - Sante, 21 - Laimdoty, 22 - Pribrezhny. *S. demissium, S. phureja, S. verrucosum, S. sparsipilum* and *S. vernei* are presented by two PCR biotypes. Electrophoresis in 1,8% agarose gel in TAE buffer.

the lower part of a pattern of Izora variety and which coincided in size with a similar fragment from *S. demissum*, hybridized with *S. demissum* (data not shown). And again, this not surprising because *S. demissum* was used in the development of the Izora variety. A fragment from Sante variety with similar size hybridized with four (of six) varieties of *S. tuberosum*, but not with any species analyzed (Figure 3B). Probably, the relative which gave this fragment to the genome of Sante was not included in the species analyzed.

It is interesting to point out the pattern revealed in the analysis of wild potato species. *Solanum sparsipilum* was represented by 9 plants, which were divided into 2 groups (PCR biotypes) (Figure 2). Patterns of these 2 biotypes in the hybridization with *S. tuberosum*, used as a labeled probe, were very similar (Figure 3A), but in the hybridization with *S. vernei*, they were very different and one of the biotypes was similar to the *S. vernei* pattern (data not shown). Therefore, DNA sequences of *S. vernei* are present in the genome of one of the plants of *S. sparzipilum*. In general *S. sparzipilum* is probably not a genetically homogenous species and contains hybrids. A hybrid nature of the *S. microdontum* species was also discovered in at least one of the plants. We think this plant is a hybrid which contains a genetic material from the genome of *S. vernei*.

These examples demonstrate the suitability of UP PCR technique for solving problems of taxonomy and verifying of seed purity of collection potato samples. It is obvious that this approach may be used in analysis of genome composition of varieties of *S. tuberosum*, in solving the problems of the origin of cultivated potatoes and in investigating genetic relationships of potato species.

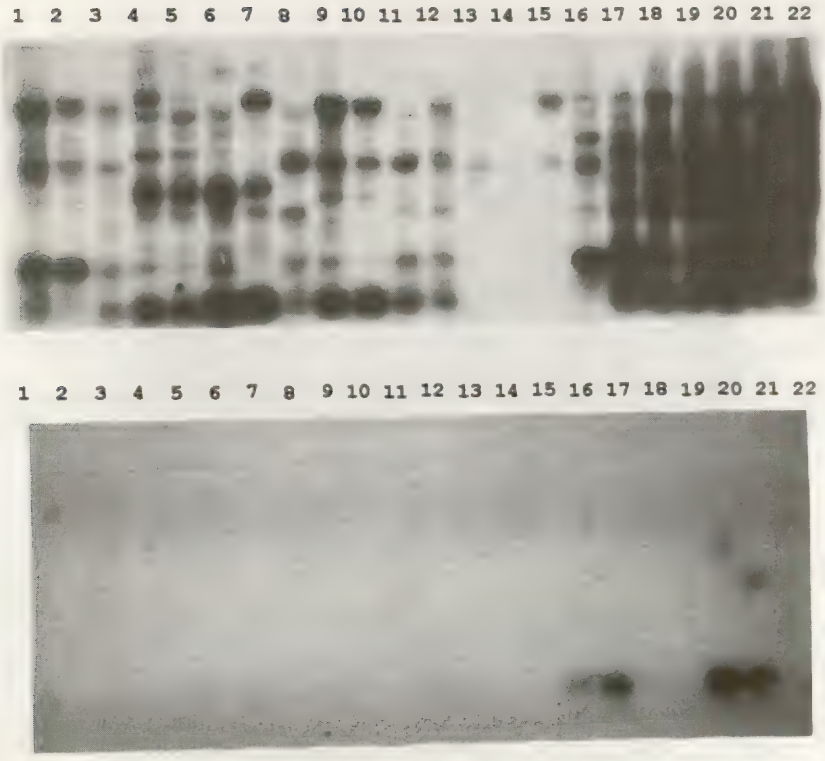


Figure 3. Blot hybridization of amplified DNA (primer 0.3-1) of potato varieties and species. A. (top) Hybridization with labeled DNA of Sante variety. B.(bottom) Hybridization with labeled eluting fragment of UP-PCR pattern of Sante variety. Gel, used for blotting, is given in Figure 2.

The UP PCR technique is very useful for genome analysis of interspecific and intergeneric hybrids of different organisms (Bulat et al., 1993). We were able to definitely prove, on DNA level, the hybrid nature of somatic hybrids obtained by the protoplast fusion of *Lycopersicon esculentum* and *Solanum etuberosum* (Gavrilenko et al., 1992). UP PCR patterns of hybrids obtained with the use of three different universal primers are given in Figure 4. As it can be seen, they are "recombinant" forms of the patterns of parents, i.e., fragments of patterns of parents in various combinations for each primer enter in the patterns of hybrids. Intergeneric somatic hybrids between *L. esculentum* Pl-alb 1 and *S. etuberosum* (b 3 and g 1) (see Figure 4) could not be distinguished using the three primers. The hybrid 1 d from the fusion combination between *L. esculentum* cv.*Tamina* and (*S. brevidens* X *S. etuberosum*) had a slightly different pattern that was probably caused by the contribution of an additional parent in the hybrid genome. To prove the presence of parental DNA in the patterns of somatic hybrids, cross dot hybridization of amplified DNA of parents and hybrids with labeled amplified DNA of one or another parent was carried out (Figure 5). It is obvious that both parents contributed approximately equal parts to the genome of somatic hybrids b 3 and g 1. This fact corresponds to their amphidiploid nature (Gavrilenko, personal comm.).

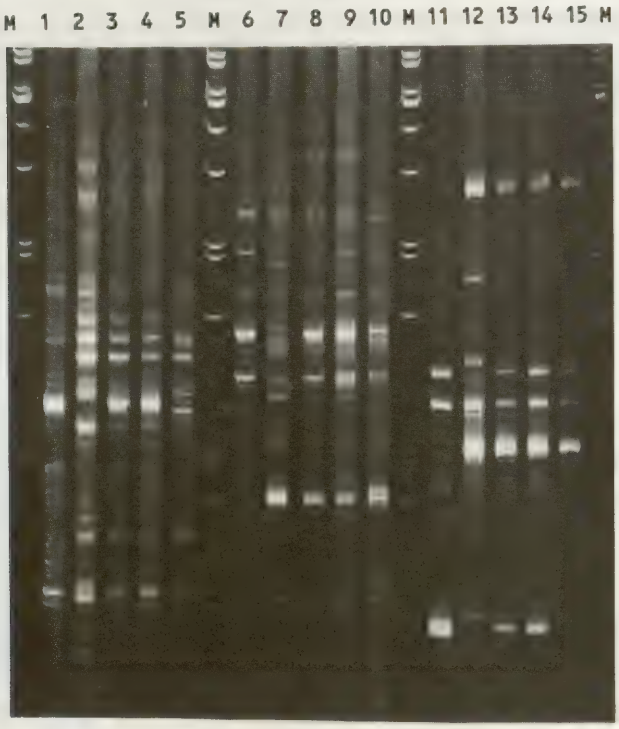


Figure 4. UP PCR analysis of somatic hybrids between *Lycopersicon esculentum* and *Solanum etuberosum*. Lanes 1,6,11 - *L. esculentum* Pl-alb 1 (plastomic mutant); 2,7,12 - *S. etuberosum*; 3,8,13 and 4,9,14 - somatic hybrids b-3 and g-1 from fusion combination *L. esculentum* Pl-alb 1 + *S. etuberosum*; 5,10,15 - somatic hybrid from fusion combination *L. esculentum* cv. Tamine + (*S. brevidens* x *S. etuberosum*). Lanes 1-5 - primer number 45; 6-10 - primer number 3-2 and 11-15 - primer number 0.3-1.

As to the hybrid I d (hexaploid), it did not differ from other hybrids in homology of amplified DNA and contained those parental genomes. The third genome of *S. brevidense* in fact did not influence the results of hybridization and this corresponds with our data about the close relationships of *S. brevidense* and *S. etuberosum* on the DNA level (unpublished data).

Data presented here demonstrate that DNA which is amplified with one primer does not hybridize with DNA amplified with another primer (i.e., hybridizational primer-specificity, Bulat and Mironenko, 1992). This effect appears even for universal primers with similar sequences. For example, 16-mer primers 45 and 3-2, used in our work, have identical 6 nucleotides at the 3' ends.

Thus, UP PCR technique is reliable for verification of the hybrid nature and is more informative as compared with hybridization data of separate parental genes (Gavrilenko et al., 1992).

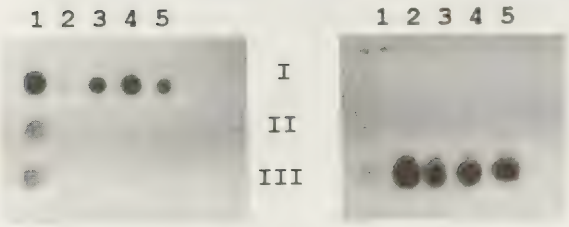


Figure 5. Hybridization analysis of somatic hybrids by the method of dot hybridization amplified in UP PCR DNA. A. Hybridization of DNA of parents and somatic hybrids amplified with 3 various primers with labeled amplified DNA (primer 45) of *L. esculentum* Pl-alb 1. B. Hybridization of the same DNA with labeled amplified DNA (primer 0.3-1) of *S. etuberosum*. 1 - *L. esculentum* Pl-alb1; 2 - *S. etuberosum*; 3 - somatic hybrid g-1; 5 - somatic hybrid 1-d. I - primer 45; II - primer 3-2; III - primer 0.3-3.

GENOTYPING AND ANALYSIS OF GENETIC RELATIONSHIP OF WHEAT SPECIES AND ITS RELATIVES

Protein markers are usually used in genome analysis in genotyping and mapping of wheat and its relatives (Konarev et al., 1993). At present, for these purposes attempts were undertaken to use RFLPs. RFLP-analysis with repetitive DNA may be successfully used for genotyping and elucidation of genetic relationships between wheat and *Aegilops* species (Strelchenko et al., 1992; Talbert et al., 1992; Zhang and Dvorak, 1992). However, the analysis of intraspecific polymorphism by this method is very difficult due to a genetic homogeneity of these species (Sorrells, 1992).

In our work we tried to use UP PCR for solving the above mentioned problems. Plant material of wheat and other Triticeae species was taken from the VIR collection (Table 2).

Methods used here are as the ones mentioned above. The first analysis was done to verify the possibility of using this technique to distinguish varieties of bread wheat. The following varieties of various breeding programs were taken: Chinese Spring, Diamant, Foton, Leningradskaja 90 and H-8810/47. Primers were chosen that maximally separated *Triticum aestivum* from *T. monococcum*. As a result, we chose a primer with random sequence AS15. Results of PCR analysis of the 4 varieties of *T. aestivum* as well as *T. dicoccum*, *T. monococcum* and *Hordeum vulgareare* are presented in Figure 6. Most differences were obtained between the most distant species of the Triticeae tribe - wheat and barley.

Patterns of most distant species of *Triticum* genera *T. aestivum* and *T. monococcum* were quite distinct. It should be noted that the pattern of *T. monococcum* was not completely reproduced in patterns of polyploid wheat. This may indicate divergence of their A genomes. Smaller but reliable differences between patterns of *T. aestivum* and *T. dicoccum* as well as between various wheat varieties were observed. Moreover, a complex pattern may indicate the presence of an additional genome in *T. aestivum*. These data confirm close relationships of *T. dicoccum* and *T. aestivum* (MacKey, 1977; Talbert et al., 1992). The wheat varieties are represented by

Table. 2. List of wheat and its relatives used in analysis.

Species	VIR catalogue number	Country of origin
Aegilops aucherii	K 77	unknown
A. bicornis	K 1327	Egypt
A. longissima	K 202	Israel
A. searsii	K 2305	Israel
A. sharonensis	K 1584	Israel
A. speltoides	K 21	unknown
A. tauschii	K 33	Turkmenia
Elytrigia intermedia	K 33706	Russia
Secale cereale var. *rossianka*		Russia
Triticum boeoticum	K 58512	Armenia
T. urartu	K 58498	Turkey
T. durum	K 41652	Russia
T. persicum	K 7882	Georgia
T. polonicum	K 1646	Germany
T. turgidum	K 28673	China
T. dicoccoides	K 20403	Israel
T. araraticum	K 40123	Iraq
T. timopheevii	K 35919	Georgia
T. aestivum cultivars		
Chinese Spring		China
Diamant		Sweden
Foton		Russia
H 8810/47		Norway
Leningradskaja 90		Russia
T. macha	K 58671	Georgia
T. spelta	K 2008	Germany
T. petropavlovskyi	K 44126	China
T. zhukovskyi	K 43063	Georgia
T. fungicidum	K 3858	Russia

two plants collected in the field (Figure 6). It is obvious that the PCR patterns of these varieties are practically indistinguishable. Nevertheless, 20 field plants of Diamant variety were verified for genetic homogeneity with two universal primers. As a result, all of them showed identical PCR patterns. Chinese Spring was also genetically homogeneous. However, plants analyzed of the Foton variety were divided into two groups. The differences were seen only in PAAG electrophoresis (data not shown). The Norwegian variety H-8810/47 was more heterogeneous. Twenty-four kernels of this variety were sprouted in the laboratory and analyzed by UP PCR method (Figure 7). In addition, seed storage protein - gliadines were also isolated and studied by PAAG electrophoresis. As a result, we found that PCR polymorphisms of this variety correlated with different electrophoretic patterns of gliadines. Thus, UP PCR technique can be used to reveal varietal differences in wheat and to carry out an active monitoring of genetic homogeneity of varieties by using a number of random DNA fragments.

We also used primer AS15 for further investigations of genetic relationships of wheat and *Aegilops* species, which were involved in evolution of bread wheat (Konarev

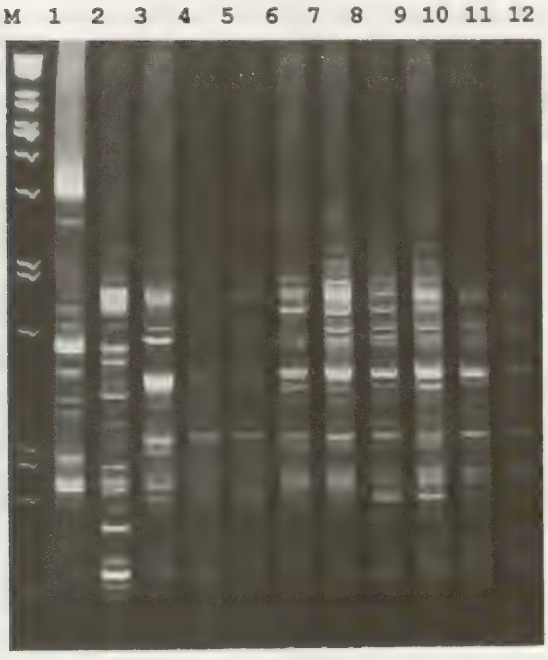


Figure 6. Genotyping of *T. aestivum* varieties by the method of UP PCR with the primer AS15. Lane 1 - *Hordeum vulgare*; 2 - *Triticum monococcum*; 3 - *T. dicoccum* and *T. aestivum* varieties: 4-5 - Foton; 6-7 - Diamant; 8-9 - Leningradskaja 90 and 10.11 - Chinese Spring. Two plants were analyzed for each variety. Electrophoresis in 5.5% PAAG in TBE buffer.

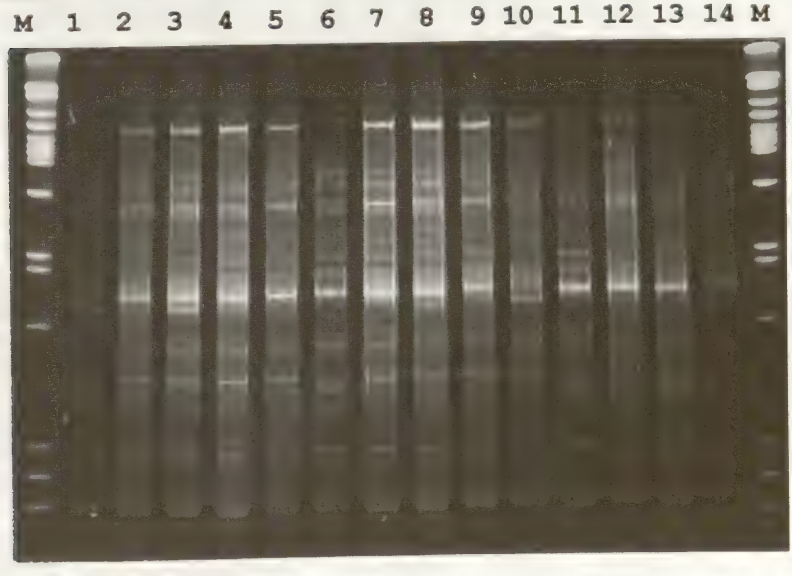


Figure 7. Heterogeneity of *T. aestivum* variety H-8810/47 on UP PCR patterns with using the primer AS15. Lanes 1-14 - sprouts of a variety H-8810/47.

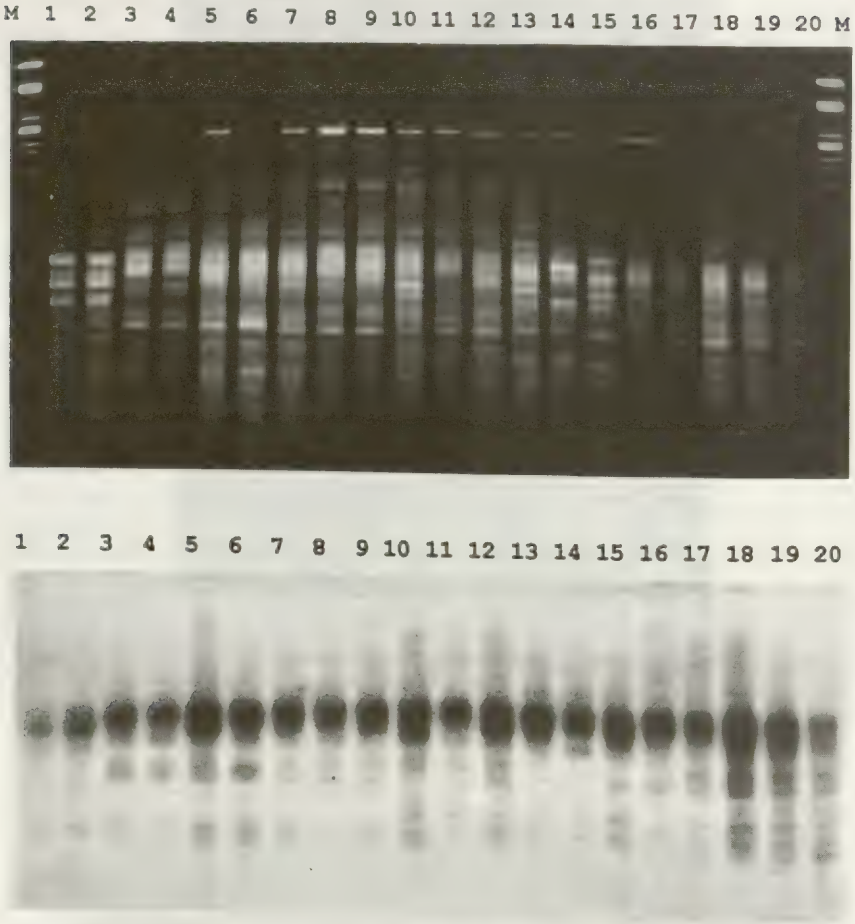


Figure 8. Genotyping of *Triticum* species by UP PCR technique with the primer AS15. A.(top). PCR patterns obtained in agarose gel. Lane 1 - *T. urartu;* 2 - *T. boeoticum;* 3-4 - *T. durum;* 5 - *T. polonicum;* 6 - *T. persicum;* 7 - *T. turgidum;* 8-9 - *T. dicoccoides;* 10 - *T. spelta;* 11 - *T. macha;* 12 - *T. petropavlovskyi;* 13 - *T. timopheevii;* 14 - *T. araraticum;* 15 - *T. zhukovskyi;* 16-17 - *T. fungicidum* and *T. aestivum* varieties; 18 - Chinese Spring; 19 - Damant; 20 - Leningradskaja 90. B. (bottom) Blot hybridization of amplified DNA of wheat species with labeled amplified DNA of *T. aestivum* variety H-8810/47 (a representative of a major type of polymorphism - see Figure 7) as a probe. For blotting, the gel (Figure 8a) was used.

et al., 1993). Data on PCR genotyping of these species are presented in Figure 8. As can be seen, diploid wheat species gave similar PCR patterns, which are different from those for polyploid wheat.

This could indicate the separation of *Triticum* genera. Analysis of the polyploid wheat - *T. spelta* (genomes ABD), *T. timopheevii*, *T. araraticum* (genomes AG) and *T. zhukovskyi* (genomes AAG) distinguished these genomes. Thus, UP PCR genotyping allows one to discern wheat of various genome composition. Hybridizational analysis of

the tested wheat species for amplified DNA (Figure 8B), indicates that there is a close genetic relationship between all polyploid wheat species.

On the contrary, an analysis of PCR patterns of *Aegilops* species indicates that there is greater divergence of the tested genomes. The PCR pattern of *A. searsii* showed greatest similarity with the pattern of soft wheat. This corresponds to some of the published data (Kerby et al., 1990; Konarev et al., 1993). The PCR patterns of *A. longissima* and *A. sharinensis* were also related as well to *A. speltoides* and *A. aucherii*. At the same time, it should be stated that there is a discordance of patterns of *A. tauschii* and *T. aestivum*. This could be related to the possibility of intraspecific polymorphism in *A. tauschii* or with the divergence of genome D in the genotype composition of soft wheat. Hybridizational analysis of *Aegilops* species studied (Figure 9A,B) indicates that there is a close relationship in these species and the wheat species (Figure 8A,B). This corresponds to published data of some authors (MacKey, 1977; Kimber and Sears, 1983). The representatives of other genera *(Secale cereale* and *Elytrigia intermedia*) gave very weak hybridizational signals. This testifies that there is less resemblance of their genomes with genomes of wheat and *Aegilops*.

After eluting of the separate fragments of UP PCR patterns and using them as hybridizational probes or substrates for forming heteroduplexes (Orita et al., 1989; White et al., 1992), it is possible to reliably determine the carriers of various wheat genomes and to discover details concerning the structure and evolution of these genomes.

It should be pointed out that a number of species analyzed showed UP PCR polymorphisms, which obviously reflects a genetic heterogeneity of studied samples. That is why samples of these species were represented by 2 or 3 plants. For example, two plants of the heterogeneous sample of *T. durum* were slightly distinguishable in PCR patterns (Figure 8A). Three plants of *A. aucherii* (Figure 9A) behaved in a similar manner. So, to choose the material it is necessary to conduct preliminary UP PCR analysis of samples and species concerning heterogeneity.

CONCLUSION

We have demonstrated here the usefulness of UP PCR technique for solving problems of the origin of polyploid species and varietal identification of wheat.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the active assistance of Marina Usoltcheva, Nikolai Vlasov and Tatjana Gavrilenko for supplying somatic hybrids and for detailed discussion of the manuscript. The plant material was kindly provided by Tuber Crops and Wheat Depts. of VIR. This work was partly supported by funds from the Russian State program "Frontiers in Genetics."

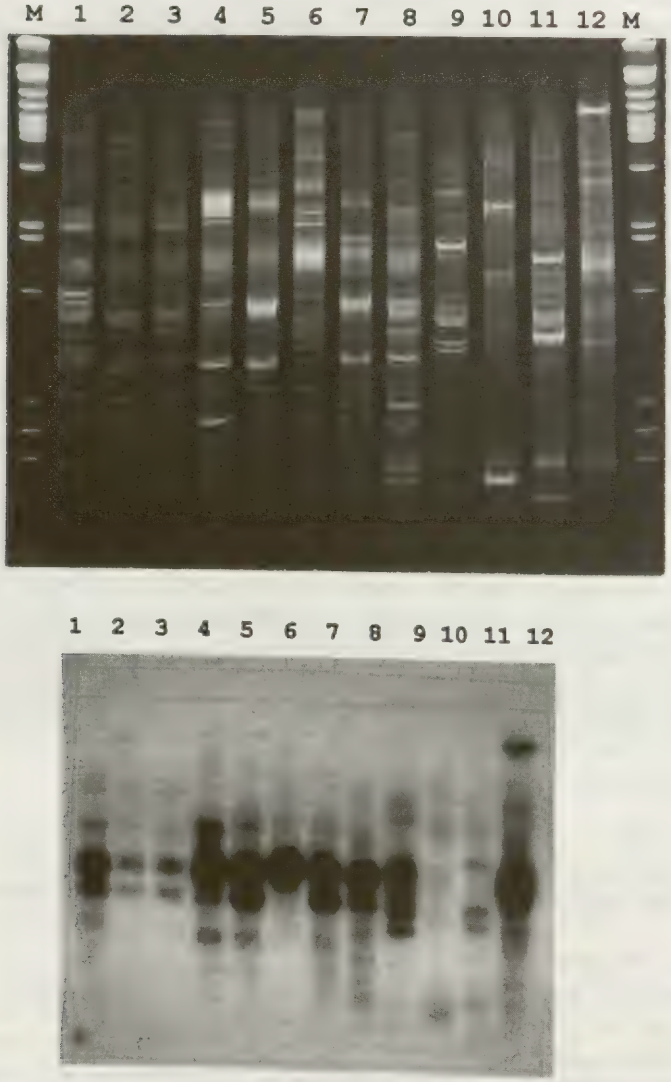


Figure 9. Genotyping of *Aegilops* species by UP PCR technique with the primer AS15. A. (top). Patterns obtained in PAAG gel. Lanes 1-3 - *A. aucherii*; 4 - *A. bicornis*; 5 - *A. longissima*; 6 - *A. searsii*; 7 - *A. sharonensis*; 8 - *A. speltoides*; 9 - *A. tauschii;* 10 - *Elytrigia intermedium*; 11 - *Secale cereale*; 12 - *T. aestivum,* variety H-8810/47.
B. (bottom) Blot hybridization of amplified DNA of *Aegilops* species with labeled amplified DNA of *T. aestivum* variety H- 8810/47 as a probe. For blotting the agarose gel, the same arrangement of samples (Figure 9A) was used.

REFERENCES

Bulat, S.A. and Mironenko, N.V. 1990. Species identity of the phytopathogenic fungi *Pyrenophora teres* Drechsler and *P. graminea* Ito and Kuribayashi. Mikologia i Phytopathologia (Leningrad, U.S.S.R.) 24:435.

Bulat, S.A. and Mironenko, N.V. 1992. Polymorphism of yeast-like fungus *Aureobasidium pullulans* (De Bary) revealed by universally primed polymerase chain reaction: species divergence state. Genetica (Moscow) 28:4-19.

Bulat, S.A., Filatov, M.V. and Pantina, R.A. 1993. Detection of human DNA in cell hybrids by the universally primed polymerase chain reaction (UP-PCR) technique: species-specificity of amplified DNA. Cytologia (St. Petersburg, Russia) 35: (in press).

Bulat, S.A., Kaboev, O.K., Mironenko, N.V., Ibatullin, P.M., Luchkina, L.A. and Suslov. V. 1992a. Universal primed polymerase chain reaction: primer and amplified DNA characteristics. Genetica (Moscow, Russia):28:5-19.

Bulat, S.A., Mikhailov, N.V. and Korolyuk, A.M. 1991. Gene identification of bacterial species and serovars by the method of polymerase chain reaction with universal oligonucleotides: re-identification of previously isolated *Yersinia pseudotuberculosis* strains. J. of Microbiol., Epidemiol. i Immunobiol. (Moscow, Russia) 12:2.

Bulat, S.A., Mironenko, N.V., Zharova, V.P. and Nagornaya, S.S. 1992b. Species identification of fungi by polymerase chain reaction method with universal oligonucleotides. Genotyping of taxonomic species *Issatchenkia orientalis* Kudriavzev and *Candida krusei* (Cast.) Berkhout. Cytologia i Genetica (Kiev, Ukraine) 26:2-25.

Bennett, M.D. and Smith, J.B. 1991. Nuclear DNA amounts in angiosperms. Phil. Trans. R. Soc. Lond. 334:309.

Britten, R.J. 1986. Rates of DNA sequence evolution differ between taxonomic groups. Science 231:1393.

Drake, J.W. 1992. Mutation rates. BioEssays 14:137.

Echt, C.S., Erdahl, L.A. and McCoy, T.J. 1992. Genetic segregation of random polymorphic DNA in diploid cultivated alfalfa. Genome 35:84.

Gavrilenko, T.A., Barbakar, N.I. and Pavlov, A.V. 1992. Somatic hybridization between *Lycopersicon esculentum* and non-tuberous *Solanum* species of the *Etuberosa* series. Plant Science 86:203.

Gebhardt, C., Blomendahl, C., Schachtschabel, U., Debener, T., Salamini, F. and Ritter, E. 1989a. Identification of 2n breeding lines and 4n varieties of potato (*Solanum tuberosum*, ssp. *tuberosum*) with RFLP-fingerprints. Theor. Appl. Genet. 78:16.

Gebhardt, C., Ritter, E., Debener, T., Schachtschabel, U., Walkemeier, B., Uhrig, H., and Salamani, F. 1989b. RFLP analysis and linkage mapping in *Solanum tuberosum*. Theor. Appl. Genet. 78:65.

Gorg, R., Schachtschabel, U., Ritter, E., Salamani, F., and Gebhardt, C. 1992. Discrimination among 136 tetraploid potato varieties by fingerprints using highly polymorphic DNA markers. Crop Sci. 32:815.

Grun, P. 1990. The evolution of cultivated potatoes. Econ. Bot. 44(suppl.):39.

Halward, T.M., Stalker, H.T., LaRue, E.A. and Kochert, G. 1991. Genetic variation detectable with molecular markers among unadapted germ-plasm resources of cultivated peanut and related wild species. Genome 34:1013.

Hawkes, J.G. 1979. Evolution and polyploidy in potato species, *in*: "The biology and taxonomy of the Solanaceae", J.G. Hawkes, ed., Acad. Press., London.

Hedrick, P. 1992. Shooting the RAPDs. Nature 355:679.

Heusden, A.W. and Bachmann, K. 1992. Genotype relationships in *Microseris elegans* (Asteraceae, Lactuceae) revealed by DNA amplification from arbitrary primers (RAPDs). Pl. Syst. Evol. 179:221.

Kerby, K., Kuspira, J., Jones, B.L. and Lookhart, G.L. 1990. Bio-chemical data bearing of the origin of the B genome in the polyploid wheats. Genome 33:360.

Kimber, G. and Sears, E.R. 1983. Assignment of genome symbols in the Triticeae. Proc. 6th Int. Wheat Genet. Symp., Kyoto, Japan.

Konarev, V.G., Gavriljuk, I.P., Gubareva, N.K., Konarev, A.V., Peneva, T.I., and Khakimova, A.G. 1993. Genetic analysis of wheat and its relatives, *in:* "Molecular biological aspects of applied botany, genetics and plant breeding", V.G. Konarev, ed., Kolos, Moscow.

Lanham, P.G., Fennell, S., Moss, J.P. and Powell, W. 1992. Detection of polymorphic loci in *Arachis* germplasm using random amplified polymorphic DNAs. Genome 35:885.

Lapitan, N.L.V. 1992. Organization and evolution of higher plant nuclear genomes. Genome 35:171.

Li, W.-H., Tanimura, M. and Sharp, P.M. 1987. An evaluation of the molecular clock hypothesis using mammalian DNA sequences. J. Mol. Evol. 25:330.

MacKey, J. 1977. Sect. Dicoccoidea Flaksb. of wheat, its phylogeny, diversification and subdivision. Proc. Symp. "Extended Availability of Wheat Genetic Resource Bari. 5."

Nei, M. 1987. Parsimony methods, *in:* "Molecular Evolutionary Genetics", Columbia University Press, New York.

Nickrent, D.L. and Franchina, C.R. 1990. Phylogenetic relationships of the *Santalales* and relatives. J. Mol. Evol. 31:294.

Pikaard, C.S., Mignery, G.A., Ma, D.P., Stark, V.J. and Park, W.D. 1986. Sequence of two apparent pseudogenes of the major potato tuber protein, patatin. Nucl. Acid Res. 14:5564.

Orita, M., Suzuki, Y., Sekiya, T. and Hayashi, K. 1989. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874.

Reiter, R.S., Williams, J.G.K., Feldmann, K.A., Rafalski, J.A., Tingey, S.V. and Scolnik, P.A. 1992. Global and local genome mapping in *Arabidopsis thaliana* by using recombinant inbred lines and random amplified polymorphic DNAs. Proc. Natl. Acad. Sci. USA. 89:1477.

Rogowsky, P.M., Shepherd, K.W. and Langridge, P. 1992. Polymerase chain reaction based mapping of rye involving repeated DNA sequences. Genome 35:621.

Ross, H. 1986. "Potato Breeding - problems and perspectives." Verlag Paul Parey, Berlin and Hamburg.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-489.

Shattuck-Eidens, D.M., Bell, R.N., Neuhausen, S.L. and Helentjaris, T. 1990. DNA sequence variation within maize and melon: observations from polymerase chain reaction amplification and direct sequencing. Genetics 126:207.

Seino, S., Bell, G.I. and Li, W-H. 1992. Sequences of primate insulin genes support the hypothesis of a slower rate of molecular evolution in humans and apes than in monkeys. Mol. Biol. Evol. 9:193.

Sorrells, M.E. 1992. Development and application of RFLPs in polyploids. Crop Sci. 32:1086.

Strelchenko, P.P., Konarev, V.G., Antonova, O.Y., Horeva, E.V. and Lapteva, M.N. 1992. DNA polymorphism in wheat and its relatives, *in* "Conservation of plant genes: DNA banking and *in vitro* biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, New York.

Talbert, L.E., Moylan, S.L. and Hansen, L.J. 1992. Assessment of repetitive DNA variation among accessions of hexaploid and tetraploid wheat. Crop. Sci. 32:366.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acids Res. 18:7213.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res. 18:6531.

White, M.B., Carvalho, M., Derse, D., O'Brien, D.D. and Dean, M. 1992. Detecting single base substitutions as heteroduplex polymorphisms. Genomics 12:301.

Zhang, H-B. and Dvorak, J. 1992. The genome origin and evolution of hexaploid *Triticum crassum* and *Triticum syriacum* determined from variation in repeated nucleotide sequences. Genome 35:806.

THE USE OF RAPDS TO DETERMINE GERMPLASM COLLECTION STRATEGIES IN THE AFRICAN SPECIES *PHYTOLACCA DODECANDRA* (PHYTOLACCACEAE)

Tigst Demeke[1] and Robert P. Adams
Plant Biotechnology Center, Baylor University,
P.O. Box 97372, Waco, Texas 76798-7372, USA

SUMMARY

Variation in the RAPDs among African *Phytolacca dodecandra* (endod) populations and three related species was examined. The data were analyzed by principal coordinate and contour mapping of coordinate scores. RAPDs were found to be useful in detecting polymorphisms among the taxa. The RAPDs of Madagascar *Phytolacca dodecandra* were not similar to the African *P. dodecandra*, but were more closely associated with *P. icosandra* and *P. rivinoides* (South America). Four major sub-groups were discovered among *P. dodecandra* populations: Madagascar; Nigeria; Ethiopia; and southern Africa. These groupings correspond rather well with a previous study using non-polar hexane chemicals. Future germplasm collections should focus on the four regions. DNA extracted from tissue culture, seed and herbarium specimens of types 3, 17 and 44 produced polymorphic bands. The RAPDs from individual seedlings of types 3, 17 and 44 produced about 43% polymorphic bands.

INTRODUCTION

There has been a continued interest in the triterpenoid saponins of *Phytolacca dodecandra* (Phytolaccaceae), commonly called endod (Lemma et al., 1984; Parkhurst, et al., 1990). The saponins of endod possess molluscicidal, insecticidal, spermicidal, antifungal, and antiprotozoan properties. The geographical variation of *Phytolacca dodecandra* in Africa has been investigated in terms of morphology, leaf chemistry and triterpene aglycones (Adams et al., 1989, 1990; Parkhurst et al., 1990).

Analysis of total aglycone derivatives (Parkhurst et al., 1990) divided *Phytolacca* populations into a high oleanolic acid group (89%, +/-6%), and a low oleanolic acid group (53% +/- 7%). Of the three standard endod types (types 3, 17 and 44), types 3 and 44 were in the high oleanolic acid group, whereas type 17 was in the low oleanolic acid group. The molluscicidal data indicates a similar trend (Wolde-Yohannes and Kloos, 1989). It appears likely that the pathway to oleanolic acid is correlated with the production of the triterpenoid glycosides (molluscicides).

Previous analysis of the morphology (Adams et al., 1989) revealed variation both within and among regions. The first trend in the morphology was the separation of pubescent individuals from Ethiopia (E2 in Figure 1) from other populations. The second trend revealed differentiation of putative *P. dodecandra* from Madagascar (MA, Figure 1) and the third trend was the division of *P. dodecandra* into east and west African populations (Figure 1).

[1]Present address: Agriculture Canada, Research Station, P.O.Box 3000, Main, Lethbridge, Alberta, Canada T1J 4B1

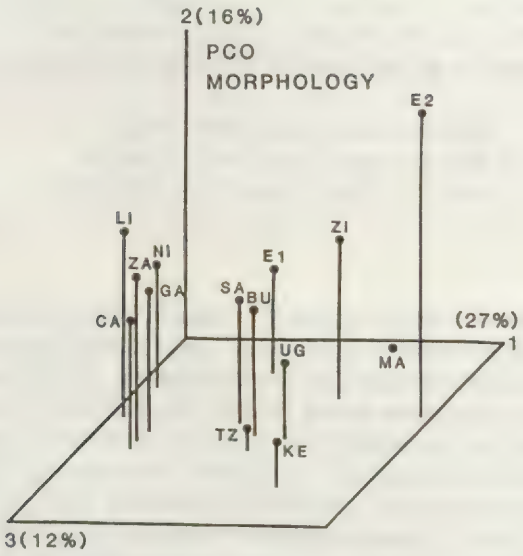


Figure 1. Principal coordinate analysis based on morphological characters of *Phytolacca dodecandra* (adapted from Adams et al., 1989). BU = Burundi and Malawi; CA = Cameroon; E1 = non- pubescent, Ethiopia; E2 = pubescent, Ethiopia; GA = Gabon; KE = Kenya; LI = Liberia, Sierra Leone & Guinea; MA = Madagascar; NI = Nigeria; SA = South Africa; TZ = Tanzania; UG = Uganda; ZA = Zaire; ZI = Zimbabwe & Zambia.

Adams et al., (1990) found the major trend in the non-polar hexane extractables to be the separation of putative *P. dodecandra* from Madagascar (MA, Figure 2) from all the African populations. The second trend found was separation of Nigeria (NI), Zambia (ZB) and Zimbabwe (ZI) populations from other African populations (Figure 2). The third trend was the differentiation of the Ghanan (GH) population (Figure 2). Aside from the separation of putative *P. dodecandra* from Madagascar, the morphology and non-polar hexane extractables did not show much correlation (cf. Figures 1 and 2).

However, taken together, the chemical and morphological data did suggest four areas for germplasm collection: north east Africa (Ethiopia, Kenya); Madagascar; southern Africa (Zambia, Zimbabwe); and east Africa (Nigeria, Ghana).

Recently, RAPDs (Random Amplified Polymorphic DNAs) have been used as molecular markers to detect genetic polymorphisms among different taxa (Williams, *et al.*, 1990). RAPDs which are generated by amplification of genomic DNA with a single primer of arbitrary nucleotide sequence produce polymorphisms that can be used as genetic markers (Caetano-Anolles et al., 1991; Demeke et al., 1992; Hu and Quiros, 1991; Williams et al., 1990).

In this work we report on DNA polymorphisms among African *Phytolacca* and related species using RAPDs. The results are compared with the morphological, chemical, and terpenoid data already available. Three other species of *Phytolacca* were included in this study. Individual endod plants of types 3, 17, 44 and from Zimbabwe were also studied to determine the extent of variability among seeds of this dioecious species.

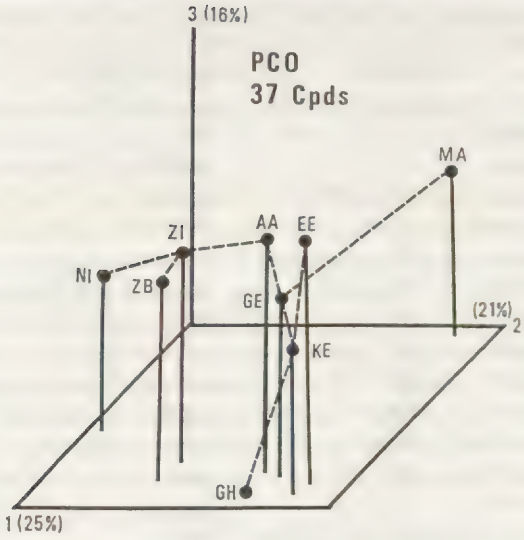


Figure 2. Principal coordinate analysis based on thirty-seven leaf chemicals for nine populations of *Phytolacca dodecandra* with a minimum spanning network superimposed (Adams et al., 1990). AA = Addis Ababa, Ethiopia; EE = Entoto Mtn., Ethiopia; GE = Guder, Ethiopia; KE = Kenya; NI = Nigeria; GH = Ghana; MA = Madagascar; ZI = Zimbabwe; ZB = Zambia.

MATERIALS AND METHODS

DNA was extracted from the plant materials (Table 1) by a modification of the SDS protocol (Dellaporta et al., 1983). PVP(0.18%) was added to the extraction buffer. DNA was also extracted from five individual seedlings of types 3, 17, 44 and from three seedlings of Zimbabwe endod to examine the polymorphisms among individuals.

Recently, RAPDs (random amplified polymorphic DNAs) have been used as molecular markers to detect genetic polymorphisms among different taxa (Williams et al., 1990). RAPDs, which are generated by amplification of genomic DNA with a single primer of arbitrary nucleotide sequence, produce polymorphisms that can be used as plants. PCR was performed in a volume of 25 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 0.01% gelatin and 0.1% Triton X-100, 0.2 mM of each dNTPs, 0.36 µM primers, 0.5 ng genomic DNA and 1 unit of TAQ DNA polymerase. A control PCR tube containing all components but without genomic DNA was run with each primer to check for contamination. Seventeen 10-mer primers (Table 2) that gave several bright bands and did not have any false bands (in the controls) were used.

DNA amplification was performed in an MJ Programmable Thermal Cycler (MJ Research, Inc.). The thermal cycle used was 94°C (1.5 min) for initial strand separation, then 40 cycles of 37°C (2 min), 72°C (2 min), 94°C (1 min). Two additional steps were used: 37°C (2 min) and 72°C (5 min) for final extension. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. The gels were photographed under UV light with Polaroid film 667. pGEM

Table 1. Sources of plant materials used in this study.

Sources of plant materials	Symbols	Sources of DNA
Phytolacca dodecandra		
Type 3, IPB (Ethiopia)	E1	seed
Type 3, tissue culture	E2	leaves
Type 17, IPB (Ethiopia)	E3	seed
Type 17, tissue culture	E4	leaves
Type 17, Adams 5290	E5	leaves
Type 44, IPB (Ethiopia)	E6	seed
Type 44, tissue culture	E7	leaves
Type 44, Adams 5289	E8	leaves
Kenya, Adams 5324	KE	leaves
Madagascar, Adams 5624	MA	seed
Zambia	ZB	seed
Zimbabwe, Adams 5595	ZI	leaves
Nigeria	NI	seed
Ghana, Adams 5559	GH	leaves
P. americana	AM	seed
P. rivinoides, Zanoni 5679	RV	seed
P. icosandra, Zanoni 5680	IC	seed

Table 2. List of primers used in this study. The RC42 was synthesized at the Plant Biotechnology Institute, NRC, Canada. The other primers (116- 247) were purchased from the University of British Colombia.

Code	Sequence (5'- 3')	Code	Sequence (5'- 3')
116	TAC GAT GAC G	218	CTC AGC CCA G
123	GTC TTT CAG G	227	CTA GAG GTC C
131	GAA ACA GCG T	232	CGG TGA CAT C
134	AAC ACA CGA G	237	CGA CCA GAG C
143	TCG CAG AAC G	239	CTG AAG CGG A
153	GAG TCA CGA G	244	CAG CCA ACC G
184	CAA ACG GAC C	247	TAC CGA CGG A
204	TTC GGG CCG T	RC42	GCA AGT AGC T
212	GCT GCG TGA C		

DNA (Promega) was used as a molecular size marker. The size of the bands and their intensity was scored as: 0= no band; 1= very, very faint; 2= very faint; 3= faint; 4= medium; 5= bright; 6= very bright band. Similarity measures were computed using absolute character state differences (Manhattan metric), divided by the maximum value for that character over all taxa (= Gower metric; Gower, 1971; Adams, 1975). Principal coordinate analysis (PCO) follows Gower (1966).

RESULTS AND DISCUSSION

Principal coordinate analysis (PCO) of RAPDs for *P. dodecandra* populations plus three other *Phytolacca* species removed 61% of the variation on the first five eigenroots (25, 12, 9, 8, 7%). Ordination of the first three axes reveals three major groups (Figure 3): complete separation of *P. americana;* a group containing *P. icosandra* (Dom. Rep.), *P. rivinoides (*Dom. Rep.) and putative *P. dodecandra* from Madagascar; a group of *P. dodecandra* populations from Africa.

The clustering of putative *P. dodecandra* (Madagascar) with the two *Phytolacca* species from the Dominican Republic raises an interesting question. This clustering may be due to non-homologous band scoring. Although Adams and Demeke (1993) successfully applied RAPDs at the section level in *Juniperus,* these *Phytolacca* species may be too distantly related for homologous band scoring.

In addition to the aforementioned three groups, it is interesting to note the clustering of the Ethiopian *P. dodecandra* (E1 - E8, Figure 3). These plants do not cluster by type. However, these types (3, 17, and 44) were based on various leaf types and growth habits. It should be noted that the types are not inbred lines but merely plants identified in the field and in test plots that have certain leaf shapes and habits.

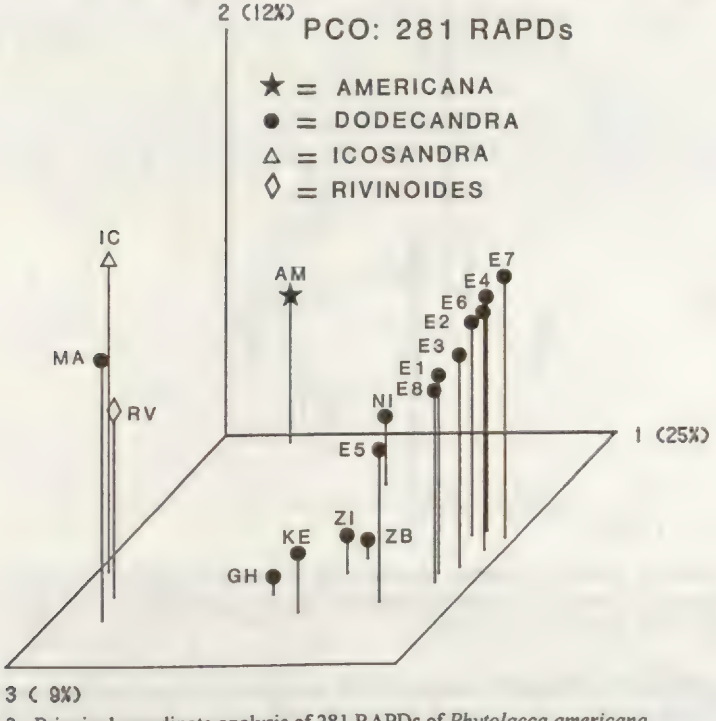


Figure 3. Principal coordinate analysis of 281 RAPDs of *Phytolacca americana, P. dodecandra, P. icosandra* and *P. rivinoides.* Note the four cluster groups (Ethiopian endod; Kenyan, South African and West African endod; *P. americana; P. icosandra, P. dodecandra* from Madagascar and *P. rivinoides.* See Table 1 for acronyms.

Because *P. dodecandra* is dioecious, one would expect to find lots of genetic diversity in these 'types' and the RAPDs show that. Controlled breeding crosses would

be needed if isogenic lines were desired. Figure 3 also shows the divergence of the three types of *P. dodecandra* (3, 17 and 44) from Ethiopia. DNA extracted from tissue culture, seed and herbarium specimens revealed several polymorphisms. The herbarium specimens were from the original clonal materials of types 17 and 44, whereas the tissue culture plants were raised from seeds collected from open pollinated plants of types 3, 17 and 44.

In order to more readily examine the trends within *P. dodecandra,* PCO was performed using just the populations of *P. dodecandra.* This resulted in four eigenroots (23.5, 19.2, 16.8, 11.4, % of variance; total = 79.4%) before the eigenroots began to asymptote. The first principal coordinate separates the Madagascar putative *P. dodecandra* (MA, Figure 4) and the second coordinate separates the Ethiopian plants E1, E3, E6, Figure 4) from other African mainland populations. The third axis shows

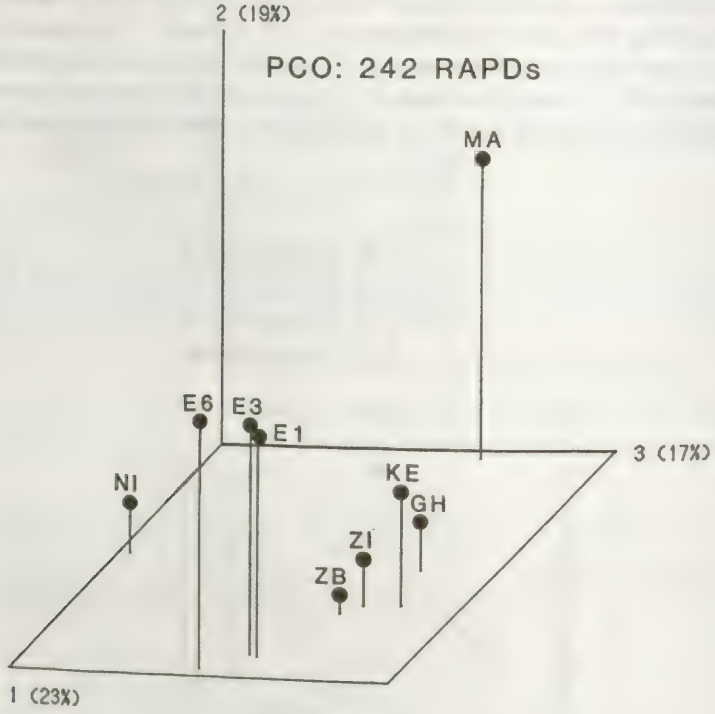


Figure 4. Principal coordinate analysis of 242 RAPDs of *Phytolacca dodecandra* from Africa. Note the separation of Madagascar *Phytolacca* from the rest of African *P. dodecandra*. See Table 1 for acronyms.

the divergence of the Nigerian plants as well as central and southern African plants. The fourth coordinate (11.4%, not shown) reveals differentiation of the Kenyan plants and the fifth axis (8.5%, not shown) separates the Ghana plants from all others.

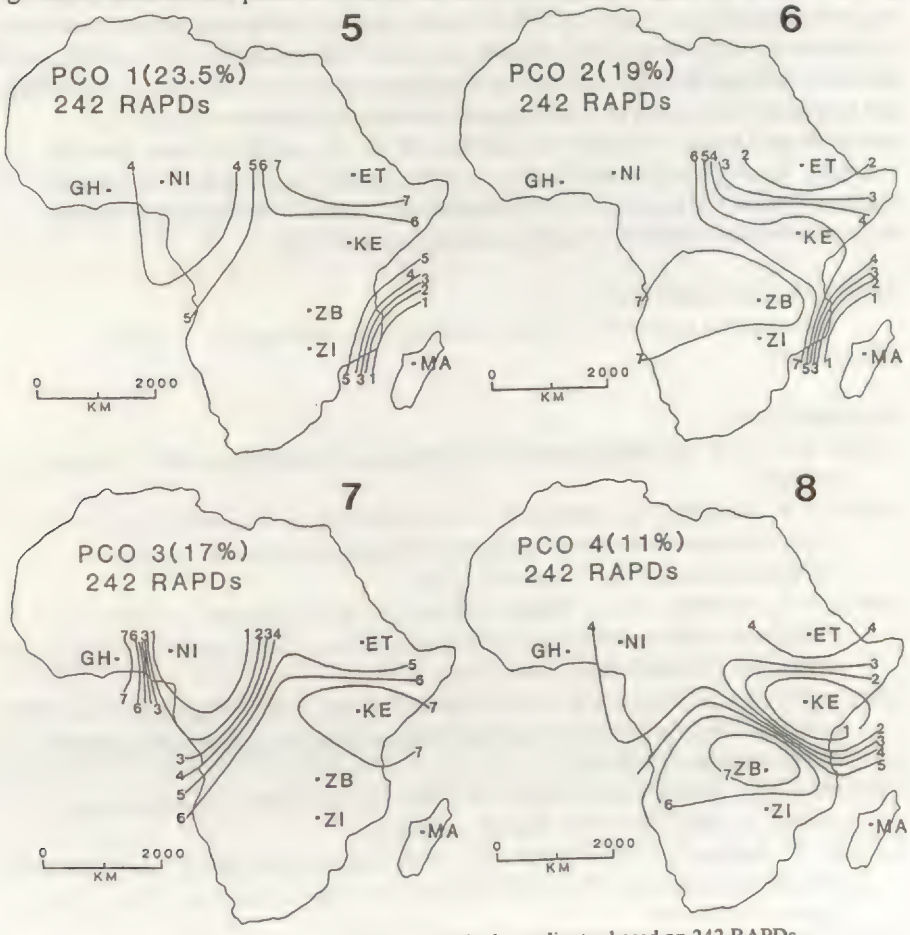
Both the previous morphological and chemical data analyses (Figures 1, 2) also separated the Madagascar *Phytolacca* from the rest of the African *Phytolacca* (Adams et al., 1989, 1990). The recognition of *P. goudotii* Briquet from Madagascar (Walter, 1909) appears to warrant possible consideration. In fact, in the previous morphological study (Adams et al., 1989) *Phytolacca* (putative *dodecandra*) from Madagascar and *P.*

goudotii were closely ordinated showing their close similarity. It seems likely that our sample of Phytolacca from Madagascar is *P. goudotii.*

The suggestion of Nowicke (1969) that the Madagascar and Ethiopian *Phytolacca* are merely ecotypic forms is not supported by our present data. Of course, it is possible that both *P. goudotii* and *P. dodecandra* co-occur on Madagascar, and we have inadvertently sampled *P. goudotii.* This is an area that deserves additional field work and analyses.

To visualize the regional trends in variation within *P. dodecandra,* the coordinate scores for each population were used as z values and the geographic locations were used as x, y values to produce contour maps for each coordinate. Figure 5 shows the divergence of the Madagascar population as well as some differentiation of the Nigeria population.

The second coordinate (Figure 6) indicates a co-differentiation of the Ethiopia and Madagascar populations. The Kenya population appears to be somewhat intermediate between Zambia and Ethiopia on this coordinate (Figure 6). The divergence of the Nigerian *P. dodecandra* plants is seen on the third coordinate (Figure 7). It is interesting



Figures 5- 8. Contour maps of the four principal coordinates based on 242 RAPDs. See text for discussion and Table 1 for acronyms.

to note the sharp difference between Nigeria (NI) and Ghana (GH) plants. The divergence of Nigeria and Ghana endod plants has also been shown by chemical analysis (Figure 2). Figure 8 shows the differences between Kenya plants and those from Zambia and Zimbabwe.

In addition, the RAPDs from five seedlings of types 3, 17 and 44 were examined and considerable variability among these plants and among the types were found (data not shown). The percentage of polymorphic bands among 5 plants of types 3, 17 and 44 was 45, 42 and 41%, respectively. *Phytolacca dodecandra* is a dioecious plant and, thus, variability among individual plants is expected. It is apparent that RAPDs will be very useful for the taxonomic evaluation of plants derived from dioecious plants. The RAPDs from the three seedlings of Zimbabwe gave only 16% polymorphic bands, indicating a closer similarity. The percentage of polymorphic bands between the three types were as follows: 3 & 17 = 57.1%; 3 & 44 = 60.3%; 17 & 44 = 55.4%; 3, 17 & 44 = 61.3%; 3, 17, 44 & Zimbabwe = 67.5%. Thus, there is a higher level of polymorphism among the types than among individual plants within a type.

Molecular markers such as RFLP and isozymes have been widely used in DNA fingerprinting and gene mapping. RFLP is very popular but it is costly, time consuming and laborious. RAPDs are much cheaper, less labor intensive and do not use radioactive chemicals and are, thus, desirable for DNA fingerprinting work. Germplasm screening and taxonomic studies are one of the major activities in germplasm centers. Morphological analysis coupled with RAPDs will be very useful for these research activities. Genetic analysis with RAPDs will be especially applicable to developing countries because of the low cost and less training needed. Conventional breeding programs will also be aided by genetic analysis using RAPDs.

ACKNOWLEDGEMENTS

This research supported by funds from Baylor University and NSF grant INT8901632.

REFERENCES

Adams, R.P. 1975. Statistical character weighting and similarity stability. Brittonia 27: 305-316.

Adams, R.P., Neisess, K. R., Parkhurst, R. M., Makhubu, L. P. and Wolde-Yohannes, L. 1989. *Phytolacca dodecandra* (Phytolaccaceae) in Africa: Geographical variation in morphology. Taxon 38: 17-26.

Adams, R.P., Parkhurst, R. M., Wolde-Yohannes, L. and Makhubu, L. 1990. *Phytolacca dodecandra* (Phytolaccaceae) in Africa. Geographical variation in leaf chemistry. Biochem. Syst. Ecol. 18:429-433.

Caetano-Anolles, G., Bassam, B. J. and Gresshoff, P. M. 1991. High resolution DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. BioTechnology 9:553-557.

Dellaporta, S.L., Wood, J. and Hicks, J. B. 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Report. 1:19-21.

Demeke, T., Adams, R. P. and Chibbar, R. 1992. Potential taxonomic use of random amplified polymorphic DNA (RAPDs): a case study in Brassica. Theor. Appl. Genet. 84:990-994.

Gower, J.C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. Biometrika 53:315-328.

Gower, J.C. 1971. A general coefficient of similarity and some of its properties. Biometrics 27:857-874.

Hu, J. and Quiros, C.F. 1991. Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Reports 10:505-511.

Lemma, A., Heyneman, D. and Silangwa, S. M. 1984. *Phytolacca dodecandra* (Phytolaccaceae). Tycooly, Dublin.

Nowicke, J.W. 1969. Palynotaxonomic study of the Phytolaccaceae. Ann. Missouri Bot. Gard. 55: 294-363.

Parkhurst, R.M., Thomas, D. W., Adams, R. P., Makhubu, L. P., Mthupha, B. M., Wolde-Yohannes, L., Mamo, E., Heath, G., Stobaeus, J. K. and Jones, W. O. 1990. Triterpene aglycones from various *Phytolacca dodecandra* populations. Phytochem. 29:1171-1174.

Walter, H. 1909. Phytolaccaceae, *in:* "Pflanzenreich IV." 83 (39).

Williams, G.K., Kubelik, A. R., Livak, K. L., Rafalski, R. A. and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Wolde-Yohannes, L. and Kloos, H. 1989. Agronomic and molluscicidal characteristics of three types of endod (*Phytolacca dodecandra*). Eth. J. Agr. Sci. 11:25-33.

RAPD FINGERPRINTING CULTIVARS OF A NATIVE FRUIT SPECIES (*AMELANCHIER ALNIFOLIA* NUTT., ROSACEAE), A NEW CANADIAN HORTICULTURAL CROP*

Brian J. Weir[1,2], Ravindra N. Chibbar[2], and Richard G. St. Pierre[1]

[1] Department of Horticulture, University of Saskatchewan, Saskatoon, SK, Canada.
[2] Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada.
* NRCC No. 36483

SUMMARY

Saskatoon (*Amelanchier alnifolia* Nutt.) cultivars are difficult to distinguish on the basis of morphological traits. A relatively new molecular technique that uses the polymerase chain reaction (PCR) to generate randomly amplified polymorphic DNA (RAPD) markers, was investigated for potential use in saskatoon cultivar identification. Thirty-eight of the forty oligonucleotide primers (9 bases long) were screened using the PCR generated amplification products. Seven of these primers generated polymorphism which was useful in distinguishing among cultivars. As few as two primers were required to uniquely characterize the seven saskatoon cultivars examined in this study.

INTRODUCTION

The saskatoon (*Amelanchier alnifolia* Nutt. Rosaceae) is native to Western Canada. It is one of about 20 closely related species of serviceberries found throughout North America. Although a saskatoon fruit resembles that of a blueberry, it is actually a pome, and therefore is closely related to the apple or pear. The saskatoon is a medium sized bush or small tree, ranging in size from about 1 to 5 meters.

Historically, the Cree Indians consumed the fresh fruit and dried them in the sun for later use in such staples as pemmican. Early European settlers also consumed the fresh fruit and preserved them in jams and jellies. Today, the fruit is eaten fresh or processed into a variety of products such as pies, jams, jellies, fruit leather, syrup, ice cream topping, wine, and chocolates.

The saskatoon is a relatively new commercial crop. Cultivation and domestication of the saskatoon began on the Canadian prairies in the early part of this century. The first two cultivars, Smoky and Pembina, were released in 1952. Today there are at least 15 cultivars, of which about ten are propagated for commercial production. Over 500 ha of saskatoon orchards have been planted in western Canada and more than 150 ha are in full production. At present, there is good financial return for the crop, and demand for fruit exceeds production (St. Pierre, 1992). It is anticipated that the industry will expand to about 4000 ha (Pruski et al., 1991).

One of the major stages in the development of any new crop, that of germplasm collection, characterization, and evaluation (Thompson, 1990), has not been adequately addressed for the saskatoon. At present, the available cultivars, which are unimproved selections from the wild, represent a small sampling of available germplasm. However, these cultivars are not easily characterized on the basis of morphological traits. In fact, within the genus, the taxonomic treatment of *Amelanchier* has been very confusing. The species within this genus are closely related and difficult to distinguish (Cruise, 1964). Jones (1946) suggested that much of the taxonomic confusion is due to variation of the

foliage, which is manifested in different developmental stages and different habitats. This variation occurs both within and between species. Specimens displaying substantial variation within any one species have often been described as new varieties or species (Jones, 1946). Consequently, accurate cultivar identification within the species *A. alnifolia* is difficult.

In other crops, molecular techniques such as isozyme analysis and RFLP markers have been utilized for cultivar identification. However, these techniques have certain disadvantages. Isozyme analysis often fails to uniquely identify any given cultivar (Bailey, 1983; Byrne and Littleton, 1989; Weeden, 1984) and can be influenced by environmental factors (Bailey, 1983). Developing RFLP probes can be costly, involves radioactive materials (Salm, 1991) and very laborious Southern blot hybridizations (Williams et al., 1990). Recently these disadvantages were overcome using the RAPD marker technique as developed by Williams et al. (1990).

Randomly amplified polymorphic DNA (RAPD) markers are generated using single, oligonucleotide primers, and the polymerase chain reaction (PCR). This reaction requires that the primer binds to genomic DNA on opposite strands of the DNA template. Binding between primer and DNA template serves as an initiation site for Taq DNA polymerase, which amplifies a DNA strand complementary to the DNA template. The priming sites must be close enough (about 2000 bp or less) for amplification of a discrete DNA product to occur. Amplification products are separated by electrophoresis in an agarose gel. The amplified DNA products are subsequently detected using ethidium bromide staining.

This technique has been used to generate genomic fingerprints to distinguish between cultivars of rice (Welsh and McClelland, 1990), corn and soybean (Williams et al., 1990), cocoa (Wilde et al., 1992), and cauliflower and broccoli (Hu and Quiros, 1991). In this report we describe the use of RAPD markers for the fingerprinting of *A. alnifolia* cultivars.

MATERIALS AND METHODS

Plant Material

The five named and two numbered cultivars of *A. alnifolia* used in this study (see Table 1) were grown in a field or glasshouse, or micropropagated. Fresh leaf tissue, including blade and petiole, was collected and washed. The leaf tissue was wrapped in foil, frozen in liquid nitrogen and stored at -80°C until further use.

Extraction and Quantification of DNA

Several methods of DNA extraction, from *Amelanchie*r leaf tissue, were examined to determine which would yield DNA suitable for the polymerase chain reaction. The method used for *Amelanchier* followed closely the CTAB method of Doyle and Doyle (1990) with the following modifications:

1. The volume of CTAB extraction buffer was increased to 10 mL and the initial incubation temperature increased to 65°C.
2. After precipitation with isopropanol, the nucleic acids were washed twice with wash buffer (70% ethanol; 10 mM sodium acetate).
3. The final precipitate of DNA was washed once with wash buffer, dried, and dissolved in filter sterile distilled water.

The amount of DNA extracted was quantified by spectrophotometric measurement of UV absorption at 260 nm. However, Doyle and Doyle (1990) have suggested that CTAB can interfere with UV absorption. A comparison of quantitative

Table 1. Saskatoon cultivars screened for polymorphism. M = leaf tissue from micropropagated plantlets. F = leaf tissue from field-grown plants. G = leaf tissue from glasshouse-grown plants.

No.	Cultivar	Material used	Cultivar Origin	Selected by	Year
1	Thiessen	M, F	Waldheim, SK	G. Krahn	1976
2	SX86-57	M, F	Unknown	Unknown	-
3	SK74-129	M, F	Braswell, SK	S. Nelson	1974
4	Pembina	M, F, G	Barrhead, AB	J. Wallace	1952
5	Honeywood	M, F, G	Parkside, SK	A. Porter	1973
6	Altaglow	M, F, G	Lacombe, AB	Unknown	-
7	Smoky	M, F, G	Peace River, AB	J. Wallace	1952

determination of DNA by UV absorption and by the minigel method of ethidium bromide fluorescence, as described by Sambrook et al. (1989), indicated that DNA estimations by UV absorption were about 10-times greater than indicated by the ethidium bromide fluorescence method (data not shown). The DNA template concentrations reported in this study were determined using the minigel method.

Primer Synthesis

Oligonucleotide primers (Table 2) were synthesized on an Applied Biosystems Model 394 DNA synthesizer using beta-cyanoethyl phosphoramidite chemistry. The oligonucleotides were purified on a Waters HPLC, using Waters Delta-Pak column (the C18 stationary phase is bonded to 5 µm spherical silica with 300 A pores). The mobile phase was 25 mM triethylammonium acetate (solvent A) and acetonitrile (solvent B), and a gradient was run for 45 min from 5% B to 22% B.

Table 2. Primers yielding distinctive DNA polymorphism. BP = unique banding pattern. UB = unique band.

Primer (Sequence)	Thiessen	SX86-57	SK74-129	Pembina	Honeywood	Altaglow	Smoky
RC-10 (AGGCGGTCT)		BP	BP				
RC-12 (GGGCAATGA)			BP		BP		
RC-15 (CGGCGTTGT)		BP					
RC-16 (GAGCGTTGT)	BP	UB					
RC-19 (TACGCACGG)						UB	
RC-21 (CAAACGCCA)			BP				
RC-30 (ACCGCTGTG)	BP UB	BP	BP	BP		BP	

Amplification Protocol

The amplification protocol developed by Williams et al. (1990) was optimized for *A. alnifolia* by standardizing a number of variables including concentrations of: $MgCl_2$ (0.5 to 7.0 mM), primer (6.25 nM to 1.0 μM), DNA template (6.25 pg to 20 ng), and reaction buffer pH (8.3, 8.7, and 9.0). The optimized conditions were used to analyze the 40 primers employed in this study.

RESULTS AND DISCUSSION

DNA Extraction

The best method of DNA extraction was the modified CTAB method (Doyle and Doyle, 1990), which consistently yielded DNA with the required purity for genetic studies involving the PCR. This method has now been optimized for extraction of DNA from fresh *A. alnifolia* leaf tissue regardless of age or growing conditions. Obtaining DNA free from contaminants is one of the most important factors in the generation of reproducible RAPD markers (Munthali et al., 1992; Weeden et al., 1992).

Amplification Conditions

The DNA concentration affected the polymorphism and intensity of staining with ethidium bromide of the amplified DNA products. Higher template concentrations (over 10 ng) usually generated more polymorphism, but also increased background smearing which made amplified products harder to distinguish clearly. Lower concentrations of template (less than 100 pg) usually decreased observed polymorphism and intensity of stained amplification products, or in some cases no amplification products were observed. Consistent banding patterns were obtained within a 100-fold range for DNA template concentration (100 pg to 10 ng).

Similarly, more polymorphism was observed at high primer concentrations (above 0.5 μM), with fewer or no observed amplification products below 0.1 μM. Primer concentration in the range of 0.1 to 0.5 μM generated clear, easily distinguishable and reproducible amplification products.

Magnesium chloride levels were examined in the range from 0 to 7 mM, with consistent amplification occurring in the range of 1.5 to 4.0 mM. Variation in magnesium chloride concentration appeared to have the most dramatic effect on the polymorphism generated. In these experiments, there were no amplification products without $MgCl_2$ in the reaction buffer, and polymorphism increased with increasing concentrations of $MgCl_2$, to a maximum of 4.0 mM. Munthali et al. (1992) reported similar results for concentration between 0 and 4 mM $MgCl_2$. At concentrations above 4.0 mM our results became variable and often no amplification products were observed.

Low pH (8.3) of the reaction buffer resulted in little polymorphism, to no observed amplification products, to extensive background smearing. Buffer pH levels of 8.7 or 9.0 generated less background smearing and therefore clearer and more distinguishable amplification products.

Based on the variables examined, the following protocol provided optimal conditions for the generation of reproducible RAPD markers. Reaction volumes of 25 μL contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 2.5 ng of genomic DNA, and 2.0 units of Taq DNA polymerase (Boehringer). The reaction mixture was overlaid with 25 μL of light mineral oil. Amplification was performed in a Hybaid DNA Thermal Reactor programmed for 1 cycle of 2 min at 94°C followed by 45 cycles of 15 sec at 94°

C, 1 min at 36°C, and 2 min at 72°C. The thermal reactor was placed in a 4°C refrigerator during amplification to speed cooling time. Amplified DNA was electrophoresed in 1.4% agarose gel containing 0.5 µg/mL ethidium bromide.

Fingerprinting Cultivars

The seven saskatoon cultivars were screened using 40 random, oligonucleotide, 9-mer primers. Thirty-eight of these primers generated amplification products. Primer base sequence composition has been shown to influence amplification ability. Williams et al. (1990) have demonstrated that primers 10 nucleotides in length require a G+C content of 40% or greater to generate detectable amplification products. A positive correlation between primer (G+C) content and amplification efficiency has been shown by Fritsch et al. (1993). The primers used in our study were all 9-mers and had either 55.5% (5/9) or 66.7% (6/9) G+C content. Although we did not examine primers with higher or lower G+C content, 95% (38/40) of our primers produced detectable amplification products. Our results tend to support the suggestion by Williams et al. (1990) that a 50 to 80% G+C content in primers is required for the generation of detectable amplification products.

However, not all of the primers that generated amplification products were useful in distinguishing among saskatoon cultivars. Some of the primers generated complex banding patterns, which made distinguishing among cultivars difficult. Other primers generated inconsistent products or gave false products (primer artifacts) in control lanes containing no DNA. In other studies it has often been necessary to screen primers to determine which may be most useful for RAPD analysis. Demeke et al. (1992), for example, screened 147, 10-mer primers before selecting 19 primers for RAPD analysis in *Brassica*. Primers generating inconsistent amplifications, no amplification, complex patterns that were difficult to interpret, and primer artifacts, have been found by other researchers (Demeke et al., 1992; Echt et al., 1992; Wilde et al., 1992). In our study we found seven primers which generated unique and reproducible polymorphism.

These seven primers generated a total of 39 medium to bright intensity, reproducible bands, which were used to distinguish saskatoon cultivars. Demeke et al. (1992) used six 9-mer primers, which generated an average of 15.5 bands per primer, as compared to 5.5 bands per primer in our study. However, Demeke et al. (1992) scored all amplification products regardless of intensity and made no comment on the reproducibility of faint bands generated in their study. Faint bands, that were generally not reproducible, were not considered as RAPD markers in our study. Similarly, Hu and Quiros (1991) used only those bands which were reproducible in multiple runs regardless of their intensity, to distinguish broccoli and cauliflower cultivars. In our study, amplification product size ranged from about 250 to 2400 bp, which is very similar to the range found in other studies (Demeke et al., 1992; Echt et al., 1992; Hu and Quiros, 1991; Vierling and Nguyen, 1992;).

The number of primers needed to distinguish cultivars in other species has been variable. Two primers were enough to distinguish 14 cultivars of broccoli, 3 primers for 12 cultivars of cauliflower (Hu and Quiros, 1991), and 1 primer for 13 genotypes of cocoa (Wilde et al., 1992). However, Halward et al. (1992) were unable to detect any polymorphism among 25 unadapted germplasm lines of cultivated peanut using 10 primers. They also indicate that this result is consistent with previous results obtained using RFLPs, and that the cause may be the narrow genetic base of the cultivated peanut.

Yet, Paran et al. (1991) were able to distinguish near isogenic lines of lettuce using 212 primers, which suggests that genetic polymorphism in the peanut germplasm

might be detected if more primers had been screened. In our study, we could distinguish among all seven genotypes with as few as two primers. Primer RC-30 uniquely characterized cultivars 1,2,3,4 and 6, and primer RC-12 distinguished among cultivars 5 and 7 (see Figure 1).

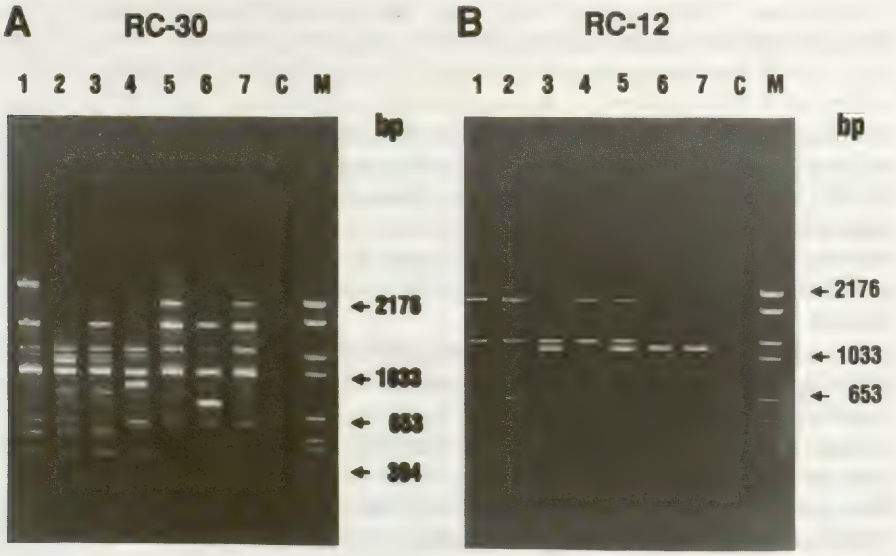


Figure 1. Random amplifications of DNA segments from seven cultivars of *A. alnifolia* were generated following the PCR amplification protocol described in this study. Numbers above each lane correspond to individual cultivars as indicated in Table 1. Genomic DNA was omitted in reactions (lane C) to indicate the presence of primer artifacts. DNA molecular-weight marker VI (Boehringer) appears in lane M. Polymorphism generated using primer RC-30 (ACCGCTGTG) uniquely characterizes five of the seven cultivars (Figure 1a). The remaining two cultivars can be distinguished from the polymorphism generated using primer RC-12 (GGGCAATGA) (Figure 1b).

CONCLUSIONS

The generation of reproducible, RAPD markers using 9-mer primers to distinguish among seven saskatoon cultivars is possible following our protocol for DNA extraction and amplification. Thirty-eight of forty primers screened generated amplification products, of which seven generated polymorphism useful in cultivar characterization. As few as two primers were enough to distinguish among the seven cultivars. Based on the genetic polymorphism observed in this study, it is anticipated that distinguishing among all commercial saskatoon cultivars will be possible.

ACKNOWLEDGEMENTS

Brian Weir is a grateful recipient of a University of Saskatchewan, College of Graduate Studies and Research Scholarship. Part of this work was funded by the Native Fruit Development Program, Agriculture Development Fund, Saskatchewan Agriculture and Food. Mr. D. Schwab, DNA Services Unit, PBI, is gratefully acknowledged for synthesizing the primers.

REFERENCES

Bailey, D.C. 1983. Isozymic variation and plant breeders' rights, *in*: "Isozymes in Plant Genetics and Breeding." S.D. Tanksley and T.J. Orton, eds., Elsevier Science Publishers, B.V., Amsterdam.

Byrne D.H. and Littleton, T.G. 1989. Characterization of isozyme variability in apricots. J. Amer. Soc. Hort. Sci. 114(4):674-678.

Cruise, J.E. 1964. Studies of natural hybrids in *Amelanchier*. Can. J. Bot. 42:651-663.

Demeke, T., Adams, R.P. and Chibbar, R. 1992. Potential use of random amplified polymorphic DNA (RAPD): a case study in *Brassica*. Theor. Appl. Genet. 84:990-994.

Doyle, J.J. and Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12(1):13-15.

Echt, C.S., Erdahl, L.A. and McCoy, T.J. 1992. Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa. Genome 35:84-87.

Fritsch, P., Handson, M.A., Spore, C.D., Pack, P.E. and Rieseberg, L.H. 1993. Constancy of RAPD primer amplification strength among distantly related taxa of flowering plants. Plant Mol. Bio. Reporter 11(1):10-20.

Halward, T., Stalker, T., LaRue, E. and Kockert, G. 1992. Use of single-primer DNA amplifications in genetic studies of peanut (*Arachis hypogaea* L.). Plant Mol. Bio. 18:315-325.

Hu, J. and Quiros, C.F. 1991. Identification of broccoli and cauliflower with RAPD markers. Plant Cell Rep. 10:505-511.

Jones, G.N. 1946. American species of *Amelanchier*. Illinois Biological Monographs, University of Illinois Press, Urbana, Illinois. Vol. XX(2).

Munthali, M., Ford-Lloyd, B.V. and Newbury, H.J. 1992. The amplification of polymorphic DNA for fingerprinting plants. PCR 1(4):274-276.

Paran, I., Kessile, R. and Michelmore. R. 1991. Identification of restriction-fragment-length-polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines. Genome 34:1021-1027.

Pruski, K., Mohyuddin, M. and Grainger, G. 1991. Saskatoon (*Amelanchier alnifolia* Nutt.), *in:* "Biotechnology in Agriculture and Forestry. Vol. 16, Trees III," Y.P.S. Bajaj, ed., Springer-Verlag, Berlin, Heidelberg.

Salm, P. 1991. Molecular markers and detection of qualitative traits in plant improvement. Bioventure View 6(5):23-24.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

St. Pierre, R.G. 1992. The development of native fruit species as horticultural crops in Saskatchewan. Hortscience 27(8):866 & 947.

Thompson, A.E. 1990. Breeding new industrial crops, *in*: "Advances in New Crops," J. Janick and J.E. Simon, eds., Timber Press, Portland, Oregon.

Vierling, R.A. and Nguyen, H.T. 1992. Use of RAPD markers to determine the genetic diversity of diploid, wheat genotypes. Theor. Appl. Genet. 84:835-838.

Weeden, N.F. 1984. Distinguishing among white seeded bean cultivars by means of allozyme genotypes. Euphytica 33:199-208.

Weeden, N.F., Timmerman, G.M., Hemmat, M., Kneen, B.E. and Lodhi, M.A. 1992. Inheritance and reliability of RAPD markers. Proceedings of the Symposium: Applications of RAPD technology to plant breeding. Minneapolis, Minnesota.

Welsh, J. and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18(24):7213-7218.

Wilde, J., Waugh, R. and Powell, W. 1992. Genetic fingerprinting of *Theobroma* clones using randomly amplified polymorphic DNA markers. Theor. Appl. Genet. 83:871-877.

Williams, J.G.K., Kubelik, A.R., Livak, J., Rafalski, J.A. and Tingey, S.V. 1990. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18(22):6531-6535.

CAMPTOTHECIN, AN ANTI-CANCER ALKALOID FROM *CAMPTOTHECA ACUMINATA* (NYSSACEAE)

Thomas D. McKnight and David D. Henning
Department of Biology, Texas A&M University
College Station, Texas 77843, USA

SUMMARY

Camptotheca acuminata is an example of how relatively obscure plants can perform valuable service, and why they are worth preserving. The clinical and preclinical trials of camptothecin and its derivatives as anti-cancer drugs are very promising. If the trials continue to be encouraging, these drugs will be an additional weapon in the fight against cancer. *Camptotheca acuminata* grows relatively rapidly, and it is currently not endangered; however, if demand for camptothecin increases, steps must be taken to prevent overharvesting. Once the rate-limiting steps in camptothecin biosynthesis have been identified, and the corresponding genes have been cloned, it may be possible to manipulate the pathway in transgenic cultures or trees.

INTRODUCTION

One of the frequently cited reasons for preserving plant species and germplasm is that endangered or undiscovered plants may produce novel and useful compounds, such as drugs to treat cancer or AIDS. Specific examples of recently discovered useful compounds derived from plants include the insecticide azadiractin from the neem tree (commercially available as Margasano) and the anti-cancer drug taxol isolated from the Pacific yew tree. Vinblastine and vincristine, other anti-cancer drugs that have been used clinically for many years, are derived from *Catharanthus roseus*, the Madagascar periwinkle. New anti-cancer drugs must be developed to augment current therapies, to overcome developed resistance to current drugs, to treat currently untreatable forms of cancer, and to provide more effective and less toxic alternatives to current drugs. Our research focuses on camptothecin (Figure 1), another plant-derived drug that is rapidly becoming medically important.

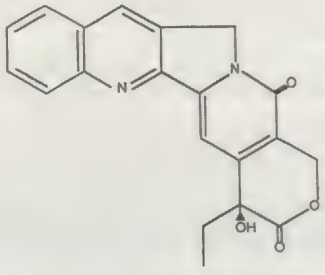


Figure 1. Structure of Camptothecin.

CAMPTOTHECIN and *Camptotheca acuminata*

Camptothecin (CPT) is produced by *Camptotheca acuminata* Decaisne (Nyssaceae), a tree that is native to southern China. This tree was introduced into the US several times during the first half of this century, but it never became widespread.

Camptotheca acuminata rapidly grows to heights of 25 meters (80 ft.), with a slender central trunk and a few branches near the top. This habit makes the tree unsuitable for ornamental purposes, and the rapid growth probably contributes to the poor quality of its wood, which makes it unsuitable for lumber (Perdue et al., 1970).

Camptothecin was discovered in the 1960s during routine screening of plant extracts for anti-cancer activity under the sponsorship of the National Cancer Institute. Extracts from the bark and wood of the tree were most effective in inhibiting the growth of several cancer cell lines, and camptothecin was identified as the active component by Monroe Wall's group at the Research Triangle Institute (Wall et al., 1966). Shortly after this finding, many of the *Camptotheca* trees in the U.S. were harvested for extraction.

Camptothecin is an indole alkaloid, as are the proven anti-cancer drugs vinblastine and vincristine, but the pathway for camptothecin production is much shorter (by approximately 40 steps) than the vinblastine and vincristine pathway. The mechanism of action of these drugs is also very different. The *Catharanthus* alkaloids inhibit the polymerization of tubulin, which, among other effects, prevents cytokinesis. CPT inhibits DNA topoisomerase I, an enzyme that can relax positive and negative supercoils in DNA. Such supercoils arise during replication of DNA and during transcription of DNA into RNA. Rapidly growing cells presumably generate more supercoils and are therefore more sensitive to topoisomerase inhibitors. Indeed, ellipticine, an inhibitor of a related enzyme, DNA topoisomerase II, has been used successfully against several cancers (Bodley and Liu, 1988).

The function of CPT in *Camptotheca* is not known, but we presume that it acts as a deterrent to animals feeding on the plant. The fact that the highest reported levels of CPT are found in the seed is consistent with an anti-feedant role.

Camptothecin has not been found in other genera (*Nyssa, Mastixia* and *Davidia*) within the Nyssaceae, although *Diplopanax*, the genus that is most closely related to *Camptotheca* (Xiang et al., 1993), has not been examined. The lack of CPT in these genera is not unusual, although it is somewhat surprising that CPT is produced by species in four divergent families: Apocynaceae, Icacinaceae, Nyssaceae and Rubiaceae. Within species of these families, however, the indole alkaloid pathway produces very different end-products. For example within the Apocynaceae, *Ervatmia heyneana* produces CPT, but *Catharanthus roseus* produces the bisindole alkaloids vinblastine and vincristine. The only common steps in these pathways are the first two, which lead to the key intermediate strictosidine.

This sporadic occurrence of CPT poses several interesting evolutionary conundrums on which we can only speculate. Apparently the CPT biosynthetic pathway is ancient, and must have occurred in an ancestor common to Nyssaceae, Apocyanaceae, Rubiaceae, and Icacinaceae. There has been a silencing of the pathway during the evolution of these families, since most of their extant species (or genera) do not produce CPT. Because the lack of CPT is widespread, its silencing probably occurred early, perhaps even before the divergence of these families. This would then require the selective reactivation of CPT biosynthesis in the few widely divergent species that produce it today. The alternative hypothesis is that the pathway remained intact during the divergence of these families and has been independently lost in almost all of their species. The selective reactivation hypothesis is more attractive since it requires fewer events. The occurrence of other secondary products in unrelated taxa has been noted by chemotaxonomists before (Cronquist, 1988).

What evolutionary forces could drive the loss and reactivation of the CPT

pathway? If CPT is an antifeedant, herbivores could eventually develop resistance (lack of uptake, degrading enzymes or a resistant topoisomerase I). In the face of such resistance, the selective value of CPT would be lost, and the pathway would be silenced eventually by accumulated mutations. Once the CPT disappeared, the selective value of resistance would be lost. Again, in a new world of sensitive herbivores, any plants that reactivated the pathway would have an advantage. Although this scenario is speculative, it can be tested by examining non-producing species from these families for the presence of DNA sequences related to specific indole alkaloid genes.

CLINICAL AND PRECLINICAL TRIALS

In early clinical trials in the U.S., CPT was usually administered as its sodium salt, and in one early trial against various advanced cancers of the gastrointestinal tract, improvement was noted in 11 of 18 patients. In subsequent studies, side effects including hematopoietic depression, diarrhea, alopecia, and urinary tract problems caused discontinuation of clinical trials. In China, where 10- hydroxycamptothecin (or its sodium salt) is used instead of CPT itself, the results have been more positive. 10-OH-CPT proved therapeutic in a phase II clinical trial on 63 patients with liver and salivary gland carcinomas. Half of the patients with primary liver carcinomas showed clinical improvement, and 40% of the patients with salivary gland carcinomas responded favorably (data reviewed by Cai and Hutchinson, 1983).

Two probable reasons have been proposed to account for the discrepancy between the results in the U.S. and China. First, 10-OH-CPT, the form most widely used in China, is much less toxic than CPT, apparently because the hydroxy derivative is excreted through the feces whereas CPT is excreted through the urine. Second, in China patients are treated with 10-OH-CPT at a much earlier stage, whereas in the U.S., CPT is usually given only after the failure of more conventional therapies (Cai and Hutchinson, 1983).

Recently, because of the Chinese experience, interest has been renewed in water soluble CPT derivatives, particularly in 7- ethyl-10-[4-(1-piperidino)-1-piperidino]-carbonyloxy- camptothecin, also known as CPT-11. This compound, developed by Daiichi Pharmaceutical Co., is highly effective against tumors *in vitro*, against human tumor xenografts in mice, and against non- small-cell lung cancer in a phase II study (Ohno et al., 1990). Apparently, CPT-11 is a pro-drug which is converted to 7-ethyl-10-hydroxy-camptothecin (also known as SN-38). SN-38, when administered directly, is more toxic and less effective than CPT- 11. Presumably the slow conversion of CPT-11 into SN-38 provides effective, and prolonged release of sub-toxic levels of the compound (Kawato et al., 1991).

SmithKline Beecham Pharmaceuticals has also developed a promising water-soluble derivative, 9-[(dimethylamino)methyl]-10- hydroxy camptothecin (SK&F 104864) that is currently in clinical trials (Kingsbury et al., 1991).

The Stehlin Cancer Research Center has reported that CPT and two of its derivatives, 9-nitro-CPT and 9-amino-CPT, are very effective in inhibiting and then killing human melanomas that have been transplanted into immuno deficient mice (Pantazis et al., 1992). The 9-amino derivative has been chosen by NIH for phase I clinical trials because it is somewhat more effective than the 9-nitro form. 9-amino-CPT is not as soluble nor as stable as the nitro derivative, and may cause problems in formulation and delivery (Kolberg, 1992).

An encouraging, but as yet unconfirmed, report from laboratories at the NIH

indicates that very low levels of CPT (400 times lower than the cytotoxic level) dramatically inhibit the productive infection of human cells by the human immunodeficiency virus (Priel et al., 1991). If these results can be repeated in a clinical setting, CPT should be useful in the treatment or prophylaxis of AIDS.

It is not clear whether the mechanism of CPT is the same in its effects on cancer cells and retrovirus production. There is no known requirement for a DNA topoisomerase in the retroviral life cycle; however, topoisomerase I activity has been found in avian sarcoma virus (Weis and Faras, 1981), equine infectious anemia virus and human immunodeficiency virus I (Priel et al., 1990). These topoisomerase I activities are distinct from those of the host cells, but their role is not clear. Topoisomerase may be required for integration into the host genome, or it may be required for the high level of transcription through the proviral DNA. In any case, the effect of CPT on retroviruses needs to be investigated further.

If clinical trials for cancer treatment continue to be encouraging, and if CPT can inhibit retrovirus replication, the demand for this drug will increase greatly. Although CPT has been synthesized (Corey et al., 1985), it is inefficient, time- consuming and costly. For the immediate future and perhaps longer, it will be more efficient to extract the drug from the trees. There have been several reports of CPT production by *Camptotheca* cells in suspension culture (Sakato and Miswa, 1974; Sakato et al., 1974; van Hengel et al., 1992). Unfortunately, these cultures produce only about 1% of the CPT found in the bark and wood of trees. The production of any CPT at all indicates that the entire pathway is functional in culture and provides hope that methods can be found to increase production. Research into the biosynthesis of CPT may provide insight into biotechnological strategies for increased production in tissue cultures or in the trees.

BIOSYNTHESIS AND MOLECULAR BIOLOGY OF CAMPTOTHECIN

Camptothecin is a monoterpenoid indole alkaloid, consisting of parts contributed by the terpenoid pathway and a modified indole ring derived from tryptophan. The pathway is presented in abbreviated form in Figure 2.

The enzyme responsible for the conversion of tryptophan to tryptamine, tryptophan decarboxylase (TDC) has been cloned from *Catharanthus roseus* (DeLuca et al., 1989), and recently a partial genomic was obtained from *Camptotheca* (R.J. Burnett and C.L. Nessler, personal comm.). The *Catharanthus* TDC gene has been expressed in transgenic tobacco plants where it directed the accumulation of tryptamine to very high levels (Songstad et al., 1990). The full-length TDC cDNA was placed under transcriptional control of the highly expressed cauliflower mosaic virus 35S promoter (CaMV 35S) and introduced into tobacco via *Agrobacterium tumefaciens*-mediated transformation. The youngest, fully expanded leaves of 39 independent CaMV 35S-TDC transformants were assayed for TDC activity and showed from 4 to 45 times greater TDC activity than tobacco controls. Not only did these plants produce enzymatically active TDC, but they also accumulated the protoalkaloid tryptamine at levels that were directly proportional to their TDC specific activity. Plants with the highest TDC activity contained over 1 mg tryptamine/gram fresh weight, which represents at least a 260-fold increase over controls. Yet in spite of their increased tryptamine content, the growth and development of the CaMV 35S-TDC plants appeared normal with no significant differences in auxin levels between high tryptamine and control plants (Songstad et al., 1990). It remains to be seen whether overexpression

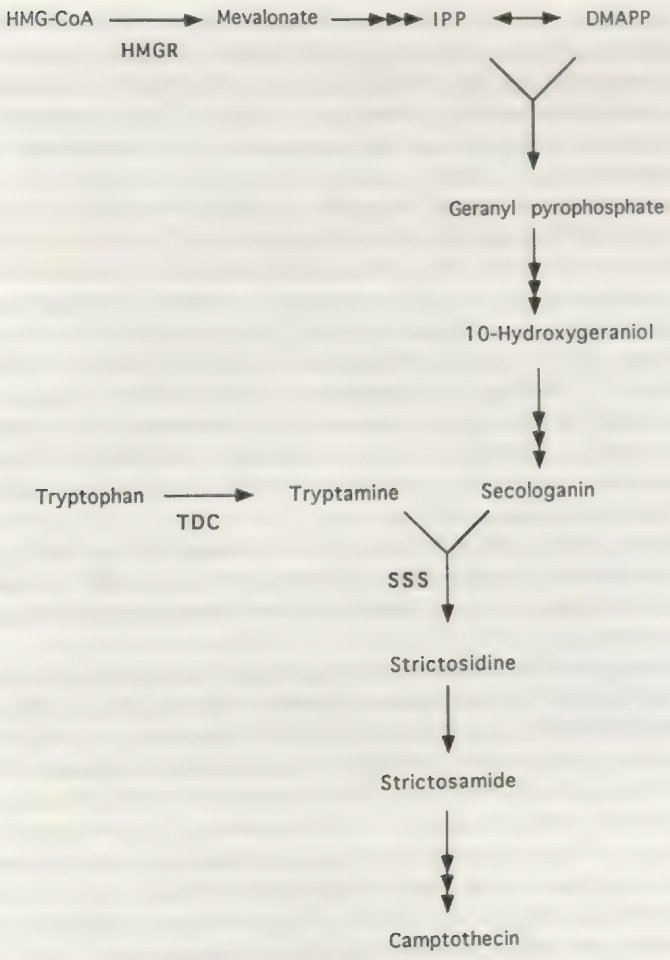


Figure 2. Key Intermediates in the Biosynthesis of Camptothecin. Synthesis of the terpenoid portion of camptothecin begins with the reduction of 3-hydroxy-3-methylglutaryl-Coenzyme A by the HMGR enzyme to form mevalonate. Mevalonate is then converted by a series of steps to isopentenyl pyrophosphate (IPP). IPP isomerase catalyzes the interconversion of IPP and dimethylallyl pyrophosphate (DMAPP). DMAPP are joined to form geranyl pyrophosphate which is also a precursor for other mono- and higher terpenes. The first step specific for monoterpene alkaloid biosynthesis is the production 10-hydroxygeraniol from geraniol (not shown). A series of steps then converts 10-OH- geraniol to secologanin, the final terpenoid precursor. Synthesis of the indole portion of camptothecin begins with the decarboxylation of tryptophan by tryptophan decarboxylase (TDC) to form tryptamine. Tryptamine and secologanin are joined by the enzyme strictosidine synthase (SSS) to form strictosidine, an intermediate common to over 1,000 monoterpene indole alkaloids in many different taxa. Strictosidine is converted to strictosamide, the last confirmed precursor of camptothecin.

of TDC in transgenic *Catharanthus* or *Camptotheca* will influence the types and amounts of alkaloids produced by cell cultures.

The monoterpenoid part of CPT is derived from the terpene pathway which begins with the reduction of hydroxymethyl glutaryl-Coenzyme-A by the enzyme hydroxymethyl glutaryl-CoA reductase (HMGR) to produce mevalonic acid. HMGR is not specific to the alkaloid pathway since the ultimate product of the mevalonate pathway is isopentenyl pyrophosphate, the starting compound for all terpenoids. Genes encoding HMGR have been isolated from a number of species, including *Catharanthus* (Maldonado-Mendoza et al., 1992) and *Camptotheca* (Burnett et al., 1993).

In most plants HMGR is encoded by a small gene family (Burnett et al., 1993; Choi et al., 1992; Chye et al., 1992). The HMGR gene cloned from *Camptotheca* is expressed in very young seedlings, and does not appear to be active in vegetative organs of older plants. Examination of the *Camptotheca* HMGR promoter region in transgenic tobacco plants showed that the gene was expressed in mature plant tissues, particularly in reproductive tissues. Wounding of the transgenic tobacco plants induced gene expression approximately 20-fold, but this induction could be suppressed by methyl jasmonate. Because of the low level of expression of this particular HMGR isogene in bark and other older tissues, it is likely that another member of the HMGR gene family is required for CPT production (Burnett et al., 1993).

The mevalonate produced by HMGR is eventually converted to geranyl pyrophosphate via isopentenyl pyrophosphate, a precursor common to all terpenoids. Geranyl pyrophosphate is then converted through a series of steps to secologanin, the ultimate terpenoid precursor of CPT.

Strictosidine, the first intermediate that contains both indole and monoterpenoid moieties, is a key intermediate and precursor to more than 1,000 terpenoid indole alkaloids, including CPT, vinblastine, strychnine, quinine and reserpine. Strictosidine is formed by strictosidine synthase, which condenses tryptamine and secologanin. Nucleic acid sequences encoding strictosidine synthase have been isolated from *Catharanthus roseus* and *Rauvolfia serpentina*, both from the Apocynaceae family (Kutchan et al., 1988; McKnight et al., 1990). Strictosidine synthase is a vacuolar enzyme in *C. roseus*, and when it was overexpressed in transgenic tobacco plants, it was correctly targeted to the vacuole there as well (McKnight et al., 1991). Transgenic tobacco plants contained up to 22 times as much strictosidine synthase activity as *C. roseus* plants.

The successful overexpression of strictosidine synthase, along with the overexpression of active tryptophan decarboxylase reported by Songstad et al. (1990), suggest that if genes encoding the rate-limiting enzymes in CPT production can be cloned, the amount of alkaloids produced by transgenic cultures or plants could be increased. We have produced transgenic *Camptotheca acuminata* suspension cultures that express high levels of strictosidine synthase from the *Catharanthus roseus* cDNA and CaMV 35S promoter, but the final levels of CPT and 10- OH-CPT were not affected by overexpression of strictosidine synthase.

We have not been able to detect strictosidine synthase activity in protein extracts of *Camptotheca acuminata*. The strictosidine synthase assays used (Eilert et al., 1987) work well for *Catharanthus roseus* tissues and for transgenic tobacco tissues expressing the *Catharanthus* enzyme, so trivial problems with the assay can be ruled out. Mixing studies where strictosidine synthase activity in transgenic tobacco extracts was measured in the presence of *Camptotheca* extracts indicated the lack of an inhibitor or further metabolism in *Camptotheca* extracts. Antibodies made in response to *Catharanthus* strictosidine synthase also fail to detect any cross reacting protein in *Camptotheca* tissues or cultures (unless they have been transformed with the *Catharanthus* gene). The

Catharanthus cDNA encoding strictosidine synthase also fails to cross react with *Camptotheca* genomic DNA and cDNAs at moderate stringencies. Despite these negative results, strictosidine synthase activity is present in many tissues since feeding studies with isolated (but intact) tissues show that almost all tissues can incorporate radioactive tryptamine into a compound that co-migrates with strictosidine in two different TLC systems (unpublished results). The failure to detect *Camptotheca* strictosidine synthase with assays and reagents designed for the *Catharanthus* system suggests that there are substantial differences in the enzyme between these plants. Such differences would be expected if the genes encoding strictosidine synthase remained dormant for significant periods, as proposed above. The eventual analysis of *Camptotheca* sequences encoding these genes will be useful in evaluating this hypothesis. It may be relevant that probes developed for tryptophan decarboxylase and strictosidine synthase from *Catharanthus* did not detect homologous mRNAs from *Cinchona* plants which produce quinine and related alkaloids (Verpoorte et al., 1993).

The intermediate immediately following strictosidine, strictosamide, is the last proven intermediate in the CPT pathway, although several plausible candidates have been isolated (Aimi et al., 1989; Carté et al., 1990). Strictosamide is derived from strictosidine via a ring closure that leads to the formation of a lactam. Radiolabeled strictosamide has been incorporated into CPT by young shoots, confirming its role as a precursor (Hutchinson et al., 1979).

Camptothecin is not the final end product of this pathway, and several naturally occurring derivatives have been described. As mentioned above, the 10-hydroxy derivative is used clinically in China and semisynthetic CPTs derivatized at the 10 position are being investigated in clinical trials as anti-cancer therapies (Fukuoa et al., 1990; Wall et al., 1990).

Chaboside, a recently reported derivative from *Ophiorrhiza pumila* (Aimi et al., 1990), is the 10-ß-D-glucoside of CPT. This compound has not been tested in clinical trials yet, but it may be an effective pro-drug that can be converted into 10-OH-CPT *in vivo*. Although chaboside is probably produced after CPT in *O. pumila* and may not be produced at all in *C. acuminata*, it may be possible to stimulate its production in cultured cells if 10-ß-D- glucose precursors can be fed to the cells. Biotransformations of this type would be an effective method for the production of novel alkaloids for anti-cancer testing. It is not yet clear whether derivatives such as chaboside and 10-OH-CPT play a specific role in the physiology or defense of the plant or whether they are merely intermediates in the degradation of CPT.

DISTRIBUTION OF CAMPTOTHECIN

Camptothecin was initially identified in extracts of the bark and wood of *Camptotheca acuminata*, and these tissues were harvested from most of the trees in California in the 1960s to provide research material (Perdue et al., 1970). Subsequent studies found that the highest levels of CPT occurred in the fruit. This finding may be useful in preventing the wholesale harvesting of trees to extract the bark and wood.

During early pre-clinical trials, a lack of anti-tumor activity was noted in some tissues (Perdue et al., 1970), but our studies have shown that every tissue examined contains CPT. Perhaps the levels of CPT were not high enough to provide activity in the assays used, or perhaps there is some antagonistic compound in these tissues. In any case, we have found CPT in all tissues and at all stages of vegetative development, although the levels of CPT vary widely.

As mentioned above, many tissues can apparently incorporate labeled tryptamine into strictosidine, but only bark and wood can produce the next compound, strictosamide. This differential incorporation of tryptamine into strictosamide, coupled with the universal occurrence of CPT suggests that at least part of the pathway is restricted to stem tissues, and that CPT or a precursor is transported to other sites.

The exact subcellular location of CPT is not known, but other indole alkaloids accumulate in the vacuole. In *C. roseus,* strictosidine synthase is a vacuolar and the bisindole alkaloids accumulate there, but there are many steps that occur in other compartments between the formation of strictosidine and the final products (DeLuca and Cutler, 1987). Verpoorte et al. (1993) point out that in *Cinchona robusta*, strictosidine synthase is strongly inhibited by quinine, and they suggest that synthesis and storage occur in different vacuolar compartments. We have isolated small vesicles from *Camptotheca* tissues that contain camptothecin, but it is not yet clear whether these represent sites of synthesis, storage, or degradation.

ACKNOWLEDGEMENTS

Research in our laboratory is supported by a grant from the National Institutes of Health (CA57592-01). We thank Dr. Craig Nessler for providing Figure 1 and for permission to cite unpublished results from his laboratory. We also thank Ronald Burnett, Ignacio Maldonado-Mendoza, and Melina Lopez-Meyer for valuable discussions and insights.

REFERENCES

Aimi, N., Nishimura, M., Miwa, A., Hoshino, H., Sakai, S. and Haginiwa, J. 1989. Pumiloside and deoxypumiloside: plausible intermediates of camptothecin biosynthesis. Tetrahedron Lett. 30:4991-4994.

Aimi, N., Hoshino, H., Nishimura, M., Sakai, S. and Haginiwa, J. 1990. Chaboside, first natural glycocamptothecin found from *Ophiorrhiza pumila.* Tetrahedron Lett. 31:5169-5172.

Bodley, A.L. and Liu, L.F. 1988. Topoisomerases as novel targets for cancer chemotherapy. Biotechnology 1315- 1320.

Burnett, R.J., Maldonado-Mendoza, I.E., McKnight, T.D. and Nessler, C.L. 1993. Expression of a 3-hydroxy-3- methylglutaryl-Coenzyme A reductase gene from *Camptotheca acuminata* is differentially regulated by wounding and methyl jasmonate. (Submitted for publication).

Cai, J.-C. and Hutchinson, C.R. 1983. Camptothecin, *in*: "The Alkaloids Vol. XXI", A. Brossi, ed., Academic Press, New York.

Carté, B.K., DeBrosse C., Eggelston, D., Hemling, M., Mentzer, M., Poehland, B., Troupe, N., Westley, J.W. and Hecht, S.M. 1990. Isolation and characterization of a presumed biosynthetic precursor of camptothecin from *Camptotheca acuminata.* Tetrahedron 46:2747-2760.

Choi, D., Ward, B.L. and Bostock, R.M. 1992. Differential induction and suppression of potato 3-hydroxy-3- methylglutaryl coenzyme A reductase genes in response to *Phytophthora infestans* and to its elicitor arachidonic acid. Plant Cell 4:1333-1344.

Chye, M.L., Tan, C.-T. and Chua, N.-H. 1992. Three genes encode 3-hydroxyl-3-methylglutaryl CoA reductase in *Hevea brasiliensis: hmg*1 and *hmg*3 are differentially expressed. Plant Mol. Biol. 19:473-484.

Corey, E.J., Crouse, D.N. and Anderson, J.E. 1985. A total synthesis of natural 20(*S*)-camptothecin. J. Organic Chem. 40:2140-2141.

Cronquist, A. 1988. "The Evolution and Classification of Flowering Plants, 2nd Ed." New York Botanical Garden, New York.

DeLuca, V. and Cutler, A.J. 1987. Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus.* Plant Physiol. 85:1099-1102.

DeLuca V., Marineau, C. and Brisson, N. 1989. Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: Comparison with animal dopa decarboxylases. Proc. Natl. Acad. Sci. USA 86: 2582- 2586.

Eilert, U., DeLuca, V., Constabel, F. and Kurz, W.G.W. 1987. Elicitor mediated induction of tryptophan decarboxylase and strictosidine synthase activities in cell suspension cultures of *Catharanthus roseus*. Arch. Biochem. Biophys. 254:491-497.

Fukuoa, M., Negoro, S., Niitani, H. and Taguchi, T. 1990. A phase II study of a new camptothecin derivative, CPT- 11, in previously untreated non-small cell lung cancer (NSCLC). Proc. Am. Soc. Clin. Oncol. 9:226.

Hutchinson, C.R., Heckendorf, A.H., Straughn, J.L., Daddona, P.E. and Cane, D.E. 1979. Biosynthesis of camptothecin. 3. Definition of strictosamide as the penultimate biosynthetic precursor assisted by ^{13}C and ^{2}H NMR spectroscopy. J. Am. Chem. Soc. 101:3358-3369.

Kawato, Y., Furuta, T., Aonuma, M., Yasuoka, M., Yokokura, T. and Matsumoto, K. 1991. Antitumor activity of a camptothecin derivative, CPT-11, against human tumor xenografts in nude mice. Cancer Chemother. Pharmacol. 28:192-198.

Kingsbury, W.D., Boehm, J.C., Jakas, D.R., Holden, K.G., Hecht, S.M., Gallagher, G., Caranfa, M.J., McCabe, F.L., Faucette, L.F., Johnson, R.K. and Hertzberg, R.P. 1991. Synthesis of water-soluble (Aminoalkyl)camptothecin analogues: Inhibition of topoisomerase I and anti-tumor activity. J. Med. Chem. 34:98-107.

Kolberg, R. 1992. Camptothecin gets a second chance in cancer trials. J. NIH Res. 4:61-64.

Kutchan, T.M., Hampp, N., Lottspeich, F., Beyreuther, K. and Zenk, M.H. 1988. The cDNA clone for strictosidine synthase from *Rauvolfia serpentina.* DNA sequence determination and expression in *Escherichia coli*. Fed. Europ. Biochem. Soc. Lett. 257:40-44.

Maldonado-Mendoza, I.E., Burnette, R.J. and Nessler, C.L. 1992. Nucleotide sequence of a cDNA encoding a 3- hydroxy-3-methylglutaryl coenzyme A reductase from *Catharanthus roseus.* Plant Physiol. 100:1613-1614.

McKnight, T.D., Roessner, C.A., Devagupta, R., Scott, A.I. and Nessler, C.L. 1990. Nucleotide sequence of a cDNA encoding the vacuolar protein strictosidine synthase from *Catharanthus roseus.* Nucleic Acids Res. 18:4939.

McKnight, T.D., Bergey, D.R., Burnett, R.J. and Nessler, C.L. 1991. Expression of enzymatically active and correctly targeted strictosidine synthase in transgenic tobacco plants. Planta 185:148-152.

Ohno, R., Okada, K., Masaoka, T., Kuramoto, A., Arima, T., Yoshida, Y. et al. 1990. An early phase II study of CPT-11: A new derivative of camptothecin for the treatment of leukemia and lymphoma. J. Clin. Oncol. 8:9907-9912.

Pantazis, P., Hinz, H.R., Mendoza, J.T., Kozielski, A.J., Williams, L.J., Stehlin, J.S. and Giovanella, B.C. 1992. Complete inhibition of growth followed by death of human malignant melanoma cells *in vitro* and regression of human melanoma xenografts in immunodeficient mice induced by camptothecin. Cancer Res. 52:3980-3987.

Perdue, R.E., Smith, R.L., Wall, M.E., Hartwell, J.L. and Abbot, B.J. 1970. *Camptotheca acuminata* Decaisne (Nyssaceae) Source of Camptothecin, an Antileukemic Alkaloid. USDA Technical Bulletin No. 1415.

Priel, E., Showalter, S.D., Roberts, M., Oroszlan, S., Segal, S., Aboud, M. and Blair, D.G. 1990. Topoisomerase I activity associated with the human immunodeficiency virus (HIV) particles and equine infectious anemia virus core. EMBO Journal 9:4167-4172.

Priel, E., Showalter, S.D. and Blair, D.G. 1991. Inhibition of human immunodeficiency virus HIV-I replication *in vitro* by noncytotoxic doses of camptothecin a topoisomerase I inhibitor. Aids Res. Hum. Retroviruses 7:65-72.

Sakato, K., Tanaka, H., Mukai, N. and Misawa, M. 1974. Isolation and identification of camptothecin from cells of *Camptotheca acuminata* suspension cultures. Agr. Biol. Chem. 38:217-218.

Sakato, K. and Misawa, M. 1974. Effects of chemical and physical conditions on growth of *Camptotheca acuminata* cell cultures. Agr. Biol. Chem. 38:491-497.

Songstad, D.D., DeLuca, V., Brisson, N., Kurz, W.G.W. and Nessler, C.L. 1990. High levels of tryptamine accumulation in transgenic tobacco expressing tryptophan decarboxylase. Plant Physiol. 94:1410-1413.

van Hengel, A.J., Harkes, M.P., Wichers, H.J., Hesselink, P.G.M. and Buitelaar, R.M. 1992. Characterization of callus formation and camptothecin production by cell lines of *Camptotheca acuminata.* Plant Cell Tiss. Org. Cult. 28:11-18.

Verpoorte, R., van der Heijden, R., Schripsema, J., Hoge, J.H.C. and ten Hoopen, H.J.G. 1993. Plant cell biotechnology for the production of alkaloids: Present status and prospects. J. Nat. Prod. 56:186-207.

Wall, J., Havlin, K., Burris, H., Weiss, G., Brown, T., Brown, J. and Kuhn, J. 1990. Phase I study of Smith, Kline and French (SKF) 104864., a novel topoisomerase I inhibitor. Proc. Am. Soc. Clin. Oncol. 9:336.

Wall, M.E., Wani, M.C., Cook, C.E., Palmer, K.H., McPhail, A.T. and Sim, G.A. 1966. Plant anti-tumor agents. I. The isolation and structure of camptothecin a novel alkaloidal leukemia and tumor inhibitor from *Camptotheca acuminata.* J. Amer. Chem. Soc. 88:3888- 3890.

Weis, J.H. and Faras, A.J. 1981. DNA topoisomerase activity associated with Rous sarcoma virus. Virology 114: 563- 566.

Xiang, Q-Y. Soltis, D.E., Morgan, D.R. and Soltis, P.S. 1993. Phylogenetic relationships of *Cornus* L. sensu lato and putative relatives inferred from *rbc*L sequence data. Ann. Missouri Bot. Gard. 80:723-734.

TRADITIONAL AND MODERN METHODS OF PLANT CONSERVATION

Daniel K. Abbiw
Botany Department, University of Ghana
Legon, Ghana

One generation passeth away,
and another generation cometh;
but the earth abideth forever.
Ecclesiastes 1:4

SUMMARY

In nature, plants normally propagate themselves. In addition, both primitive and modern man have found it necessary to conserve plants. This paper examines the different forms of plant conservation methods from ancient times to the present day. It recognizes the natural system and traditional methods (both *in situ*), and modern or scientific approaches (all *ex situ*). The successes, problems and failures of the traditional methods of plant conservation are discussed with suggested solutions to improve upon and maintain the system in modern times. The dual problems of cost and lack of technology make modern methods like DNA Bank-Net presently an unaffordable conservation technique in many developing countries. It is recommended that methods of plant conservation be adopted that are most convenient and practical in developing countries.

INTRODUCTION

Plants, like animals, reproduce to replace dead, dying or old populations for continued existence and survival of the species. There are natural mechanisms and agents to disperse fruits and seeds from the parent plant to prevent overcrowding, and ensure a wider distribution in furtherance of this survival.

Man has always been conscious of the need to conserve plants because his continued existence depends on plant life, and his activities, among other causes, tend to destroy plants. The latter is the main impediment to plant conservation. Consequently, a plant conservation program has to first address itself to the root causes of deforestation, desertification and environmental degradation, and the measures to stop or minimize these.

In many developing countries, the slash and burn system of traditional farming, with its associated shifting cultivation, is the principal cause of forest destruction and environmental degradation. Other causes include collection or gathering of fuelwood, burning of charcoal, commercial timbering, hydro-electric power generation, exploitation of mineral resources, road-building, housing and other infra-structure, industrial pollution and bush fires.

Food crops like *Zea mays* (maize) and crops like *Theobroma cacao* (cocoa) exist to this day because they have been cultivated season after season throughout the ages. In addition to crops, man relies extensively on wild plants for many of his everyday needs. For this reason, necessary measures are taken to ensure sustained yield of these wild plants. For generations, different traditional methods have been adopted--some of

these based on beliefs, symbols, signs and values--to protect wild plants from over-exploitation and extinction by all races and cultures throughout the world, because the danger of over-exploitation and extinction is greatest among wild plants.

In an heterogeneous tropical forest with its wide variety of species, about 90,000 in Latin America, 60,000 in Asia and 40,000 in Africa (including Madagascar) (Heywood, 1992), this threat is even more alarming. One man with modern machinery can tear up and level down acres of primary forest in one day that has taken centuries to establish. Many developing countries are tropical.

With civilization and the advancement of knowledge, science and technology are developing other methods of plant conservation and propagation. There is cold storage of germplasm (orthodox seeds only), tissue culture and plant cloning, then lately DNA Bank-Net to conserve plant genes in liquid nitrogen (Figure 1).

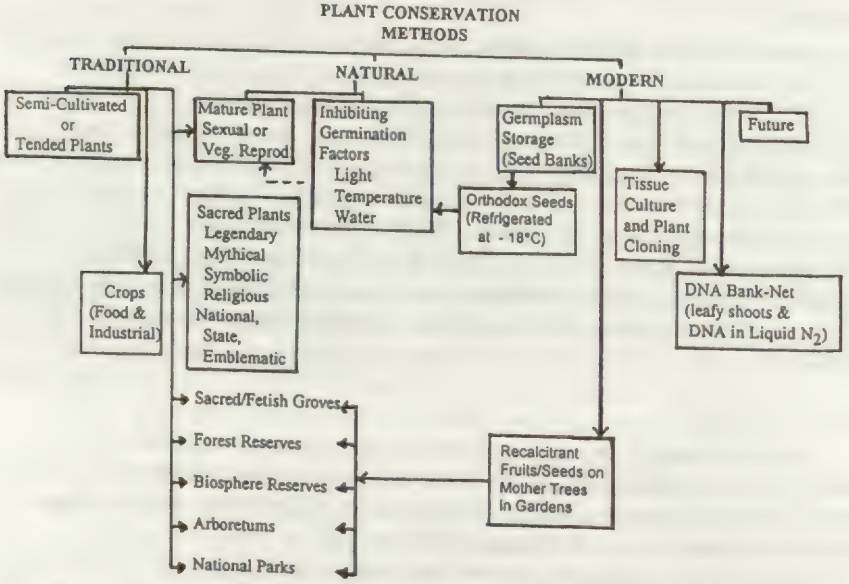


Figure 1. Summary and interactions of plant conservation methods.

THE NATURAL SYSTEM

Plant conservation by both reproduction of progeny and inhibiting the germination of seeds or fruits occur naturally.

1. *Reproduction.* Both vegetative and sexual reproduction of offspring are common in nature. In sexual reproduction, the seeds or fruits are usually first dispersed from the parent plant by one of the three main dispersal agents--water, wind and animals. Species that are efficiently dispersed and widely distributed are generally better conserved than species with limited distribution. Contrasting examples are *Ceiba pentandra*, silk cotton tree, which grows on both sides of the Atlantic, and *Turraea ghanensis*, a shrub localized only around the Akosombo Dam area in Ghana and now faced with extinction.

Naturally dispersed seeds and fruits often have: floating fruits (*Pterocarpus santalinoides*, some such fruit or seeds called drift dissemules can travel across continents - Gunn & Dennis (1979) give many examples); wings (*Petersianthus*

macrocarpus) or papas in wind-dispersed fruits and seeds (*Funtumia elastica,* West African rubber tree); hooks (*Pupalia lappacea);* sticky fruits (*Phragmanthera* and *Tapinanthus* species, Mistletoe or other attachment in animal dispersed fruits and seeds). *Desplatzia subericarpa* fruits that are eaten and generally germinated in elephant dung are included in the latter example.

Where the canopy is open, germination favors light demanders like *Lophira alata* (Red Ironwood Tree); whereas closed canopy favors shade-bearers like *Strombosia glaucescens.* Both are high forest trees.

Individuals compete for light, water and soil nutrients, with the result that only the fittest survive. This system of natural regeneration eventually leads to a climax, except for man's influence or activities.

2. *Inhibiting Seeds Germination.* There are records of seeds that have been buried under ice or in dark caves or in extremely dry conditions for many years, that have germinated with favorable conditions later. For instance, in a recent BBC program - "Words of Faith" in which the speaker equated light with life - electric lights were erected to light up dark caves to boost tourism. With these lights sprang up many seedlings that have been lying dormant over the years. Friedmann (1982) records similar examples with lichens in the Antarctic cold desert. These examples raise the question whether germplasm storage is really a modern method of plant conservation.

TRADITIONAL METHODS

Many traditional methods have been adopted to conserve plants over the years. As traditional practice is dynamic not static, necessary modifications are made to these methods, from time to time and as need be. Tradition is the oral transmission of custom, culture and beliefs from generation to generation or the practice of such belief or custom.

1. *Semi-cultivated or Tended Plants.* Wild plants that are useful to man are usually tended in and around dwelling places and on farmlands. Typical examples include *Vitellaria paradoxa* (Shea Butter Tree), *Parkia clappertoniana* (West African Locust Bean), *Dialium guineense* (Velvet Tamarind) and *Vitex doniana* (Black Plum) - all savanna trees. Examples in the high forest include *Synsepalum dulcificum* (Miraculous Berry), *Telfairia occidentalis* (Fluted Pumpkin), *Garcinia kola (*Bitter Cola), *Chrysophyllum delevoyi* (White Star Apple) and *Momordica angustisepala* (locally called 'ahensaw' in Akan and useful as a bathing sponge). With time, some tended plants may be developed into crops. In fact, all the above examples are now also being cultivated.

2. *Crops.* To meet man's needs, many useful plants are cultivated both for food and for industrial purposes. Typical food crops include *Dioscorea cayenensis* subsp. *cayenensis* (Guinea Yam), *Pennisetum americanum* (Millet), *Vigna subterranea* (Bambara Groundnut) and *Sechium edule* (Chayote). Some industrial crops are *Gossypium arboreum* (cotton), *Hibiscus subdariffa* (Roselle), *Nicotiana tabacum* (tobacco) and *Sesamum indicum* (Beniseed). Plant cultivation is an effective means of plant conservation.

3. *Sacred Plants.* Plants which are respected and feared either for their association with the fetish and juju, or are objects of worship and sacrificial offerings, or are symbols of traditional values or trees forbidden to be felled for fuelwood or medicinally important plants are believed to be sacred in folklore (Abbiw, in prep.) and accordingly venerated among many ethnic groups worldwide. These include:

a. Legendary and Mythical Plants (see Appendix I)
b. Symbolic Plants (see Appendix II)
c. Religious Plants (see Appendix III)
d. National, State or Emblematic Plants (see Appendix IV)
e. Others - neither cut nor burned (see Appendix V)

There are many other plants that are attributed with magical, juju or supernatural powers among many cultures throughout the world and are accordingly respected as such. These include *Spathodea campanulata* (Africa Tulip Tree), *Momordica charantia* (African Cucumber), *Mandragora officinarum* (Mandrake) and *Paeonia officinalis* (Common Peony), to mention a few. As traditional practices and beliefs are passed on from one generation to the next, this belief is likely to continue indefinitely.

It is worthy to note that none of the plants listed in Appendices I - V are either extinct or even threatened with extinction. This fear of the people for trees professed to have supernatural powers, and the subsequent associated taboos, eventually tends to the preservation of these species. The practice is scientifically an important conservation method and is worth support and encouragement.

Furthermore, customary laws and taboos are often shrouded in fear and superstition to enforce compliance. For instance, plants dug for their roots are covered up again with soil, allegedly to ensure the efficacy of the medicine - but it appears the practice is, in effect, a conservation measure (Abbiw, in prep.).

4. *Sacred (Fetish) Groves.* Sacred groves are most often perfectly intact relics or virtually virgin forests, in sharp contrast to the adjacent endless stretches of over-exploited secondary vegetation or abandoned and depleted farmlands (Abbiw, in prep.). Some of them were formerly burial grounds for chiefs and important people.

Hall (1978) observes that this forest type is found only in Ghana; but Burkill (1985) reports similar forests in Cote d'Ivoire and Burkina Faso; and Sanago (1983) describes sacred groves in Cote d'Ivoire, noting that there are places where moral values are taught and passed on from one generation to the next. In some areas in Cameroon, Koagne (1986) records that sacred groves are the only forested areas that remain.

It is not mere belief in any indwelling deities nor fear of punishment or even death for any violations, that has preserved such forests, but, more importantly, respect of the inherent traditional values as a cherished legacy from past generations.

However, as traditional beliefs weaken in the face of western education and religion, these groves will be increasingly endangered, and it is to be hoped that ways may be found to ensure their continued conservation (Hall & Swaine, 1981).

In Ghana, where there are about 2,500 sacred groves of all sizes, from an acre or two to five square miles, the World Bank and UNESCO under its CIPSEG (Cooperative Integrated Project on Savanna Ecosystems of Ghana) program, are assisting to access the threats facing some of these groves with the goal of eradicating these threats.

5. *Forest Reserves.* Reserves may be either protective or productive. While protective Forest Reserves are not normally exploited for timber, productive ones are exploited on the sustained yield system. There are some two hundred Forest Reserves in the forest zone of Ghana, and these together represent just over 20 per cent of the total area of the zone (Abbiw, 1990).

The percentage of the land area occupied by Forest Reserves in some other developing countries are: Sarawak, 20%; Sri Lanka, 17%; British Honduras, 11%:

Jamaica, 8%; Kenya, 3%; and India, 60% in the Himalayas, the Deecan and other mountainous areas and 20% in the plains (Taylor, 1962).

With hardly any sizable intact forests left outside Forest Reserves (in Ghana at least) - except sacred groves - these reserves appear to be the only sanctuary for the protection of the indigenous flora, especially plants that are seriously threatened with extinction.

Since Forest Reserves are governed by laws and by-laws, the success or failure of the conservation method depends both on the enforcement of these rules and regulations and their compliance or non-compliance. In many instances, illegal felling, farming and hunting, over-exploitation of merchantable species, exploitation of undersized trees, and even squatters in Forest Reserves not uncommon. Where moral laws succeeded with sacred plants and groves, legislation appears to have failed. The present management policy in many Forest Reserves in developing countries also accounts for the over-exploitation of some plants, consequent reduction in populations, leading to extinction.

For instance, while merchantable timber trees are being managed on the sustained yield system, the non-wood forest products, which constitute at least 95% of the flora, are not. Some of these products such as sweeteners, medicinal and poisonous plants, decorative plants, plants for basketry, chewing sticks and sponges are also being exploited in commercial quantities (Abbiw, 1993). In Ghana, the Chief Conservator of Forests laments that formal forest management of NTFPs (Non-timber Forest Products) is virtually nonexistent and yet urgently required (Falconer, 1992).

6. *Biosphere Reserves.* Biosphere Reserves are intended to become models of how we should live with nature. They are multipurpose protected areas established to conserve species and natural communities, and to find ways to use environments without degrading them (UNESCO MAB 1990). As of June 1990 there were 165 Biosphere Reserves worldwide.

7. *Arboretums.* This is a collection of assorted rare trees and shrubs cultivated for shade, decoration, recreational purposes, landscaping and for the aesthetic values.

The arboretum has played its part in the conservation of species like *Ginkgo biloba* Ginkgo or Maidenhair Tree. Novak (1966) records that it is a living fossil and has been planted in China and Japan since earliest times particularly near palaces and temples. The author adds that except for the attention given it by Buddhist priests, it would probably by now be extinct. However, Lanzara & Pizzatti (1977) observe that the tree has survived because it was cultivated by Chinese Monks. Bonati (1992) describes Ginkgo as a well-known vegetal fossil which has been growing on the earth crust for over 150 million years. Ginkgo is a symbol of longevity (see Appendix II).

8. *National Parks.* A National Park is primarily a sanctuary and a natural home for wildlife, where food, water, shelter and above all, security, are provided for their conservation. Among its secondary objectives include the aesthetic value and an attraction for tourists.

Since plants serve as both food and shelter for game, they play an important part in a national park. Any threat to this protection or security, that is, any threat to the forest, would naturally drive the animals away to more secure, better protected and safer environments; by protecting the forest with its numerous benefits, man would be protecting wildlife (Abbiw, 1991). In Nigeria, the establishment of the Yankari Game Reserve and the Kainji Lake National Park was the start of serious efforts for the conservation of endangered species of animals and plants (Gbile, 1992).

MODERN OR SCIENTIFIC APPROACH

The Scientific Approach consists of Germplasm Storage, Tissue Culture and Plant Cloning, and, more recently, DNA Banking (DNA Bank-Net).

1. *Germplasm Storage (Seed Banks).* There are Germplasm Storage Centers throughout the world where orthodox seeds such as *Sorghum bicolor* (Guinea Corn) and *Vigna unguiculata* (Cowpea) are stored in refrigerators at -18°C or in sealed containers. In addition to the freezing machines, an uninterrupted electricity supply is a necessary requirement.

The main centers according to the IBPGR Annual Report (1990) include the following:

CIAT	Centro International de Agricultura Tropical. Cali, Colombia
CIMMYT	Centro International de Mejoramiento de Maiz y Trigo. Mexico City, Mexico
CIP	Centro Internacional de la Papa. Lima, Peru
IBPGR	International Board of Plant Genetic Resources. Rome, Italy
ICARDA	International Centre for Agriculture Research in the Dry Areas. Aleppo, Syria
ICRISAT	International Crops Research Institute for the Semi-Arid Tropics. Hyderabad, India
IFPRI	International Food Policy Research Institute. Washington, D.C. USA
IIMI	International Irrigation Management Institute. Colombo, Sri Lanka
IITA	International Institute of Tropical Agriculture. Ibadan, Nigeria
IRRI	International Rice Research Institute. Los Banos, Philippines
ISNAR	International Service for National Agricultural Research. The Hague, Netherlands
VAVILOV	Vavilov Institute of Plant Industry. St. Petersburg, Russia

In addition to orthodox seeds, recalcitrant fruits and seeds like *Cocos nucifera* Coconut Palm and *Bertholetia excelsa* Brazil Nut are stored directly on mother trees in Sacred Groves, Forest and Biosphere Reserves, National Parks and in the Arboretums and Botanic Gardens (See Figure 1).

Root crops such as *Dioscorea bulbifera* Yam, *Coleus edulis* 'Oromo dinich' and spices such as *Zingiber officinale* Ginger and *Aframomum corrorima* Korrorima are conserved at appropriate agro-ecological areas in field gene-banks (Demissie, 1992).

2. *Tissue Culture and Plant Cloning.* These are primarily propagation methods, but any form of plant propagation is, in effect, also a conservation measure. In addition to food crops like *Xanthosoma mafaffa* Cocoyam and *Musa paradisiaca* Plantain, and cash crops such as *Elaeis guineensis* Oil Palm, the system has been successfully applied to propagate timber trees. Gbile (1992) records, "In the case of *Triplochiton scleroxylon* Obeche, this resulted in the shift in emphasis from provenance trials to the more specific and genetically productive method of clonal trials based on vegetatively produced plants. Propagules have been planted out since 1975."

3. *DNA Bank-Net.* DNA (deoxyribonucelic acid) is a chemical within the cell nucleus on which the genes are located. The biotechnology of its replication to form life as a means of conserving species has been the subject of scientific discussion in recent times.

DNA Bank-Net is an *ex situ* conservation method to store leafy shoots (DNA rich material) or extracted DNA in liquid nitrogen for subsequent utilization to supplement germplasm seed banks because only a fraction of germplasm is being presently stored. Adams (1989) observes that is partly due to lack of seeds in some species, expenses in seed collection, storage, testing and regeneration and problems with recalcitrant seeds and fruits.

The concept is a new one and the latest method of *ex situ* plant conservation. The First International Conference on DNA Bank-Net was held at the Royal Botanic Gardens, Kew, in April, 1991 (Adams & Adams, 1992).

It is expected that funds for the scientific and technological research, necessary equipment and international conferences on the subject will be readily available to enable this method of plant conservation to materialize in view of the thousands of wild species that are presently in danger of extinction.

RECOMMENDATIONS

1. Plant conservation measures must necessarily include education of the local people on the direct and indirect benefits that may be derived from the forest, and the need, therefore, to conserve it. The people must further be aware that the forest has been reserved in their own interest. Irrespective of whether it is under the forest ordinance or under native authority, there are immediate benefits.

2. The enforcement of laws and by-laws that protect the forest from any outside encroachment would be more effective with the support and participation of the local people.

3. Large areas of forest land are destroyed annually through the traditional slash and burn farming system with its associated shifting cultivation. The introduction of more scientific methods of farming such as crop rotation, planting nitrogen-fixing species and the use of farm residues for compost and manure instead of burning will reduce further encroachment on the forest and minimize the incidence of environmental degradation.

4. The present Forest Reserves Management Policy needs immediate revision. The non-wood products that are exploited in commercial quantities have to be managed on the sustained yield system like merchantable timbers.

5. In view of the useful role that the sacred groves have had from ancient times to the present in the conservation of plant species and the threat that faces some of them today, it is necessary that these relics of the forest be supported with international funds for their continued maintenance.

CONCLUSION

All the plant conservation methods - natural, traditional and modern - protect plants from extinction and the environment from degradation. The various forms, both *in situ* and *ex situ*, complement each other. In institutions where there is continuous, uninterrupted electricity supply or an alternative stand-by plant in addition to all the necessary equipment and chemicals, the technology and qualified personnel, both germplasm and leafy shoots for DNA extractions could be stored in freezers and in liquid nitrogen, respectively. Otherwise many developing countries will have to adopt plant conservation methods that are known to be traditionally more convenient, affordable and practical.

REFERENCES

Abbiw, D.K. 1990. "Useful Plants of Ghana." Intermediate Technology Publication, London, Publications Dept., Royal Botanic Gardens, Kew.

Abbiw, D.K. 1991. Detailed Inventory of Plants in Kakum National Park and Assin Attandaso Game Protection Reserve (mimeographed).

Abbiw, D.K. 1993. "Traditional and Religious Plants of West Africa." (In preparation).

Adams, R.P. 1989. DNA Bank-Net Newsletter Vol. 1, No. 1. Baylor University, Waco, TX.

Adams, R.P. and Adams, J.E. (Eds.). 1992. "Conservation of Plant Genes. DNA Banking and *in vitro* Biotechnology," Academic Press, San Diego, CA.

Bonati, A. (Ed.) 1992. "Fitoterapia," Vol. LXIII, No. 1. Milan, Italy.

Burkill, H.M. 1985. "The Useful Plants of West Tropical Africa Vol. 1." Royal Botanic Gardens, Kew.

Demissie, A. 1992. Current Status of Plant Genetic Resource in Ethiopia, *in:* "Conservation of Plant Genes. DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

Falconer, J. 1992. "Non-timber Forest Products in Southern Ghana." National Resource Institute, Kent, UK.

Friedmann, E. I. 1982. Endolithic microoganisms in the Antarctic Cold Desert. Science 215:1045-1053.

Gbile, Z.O. 1992. Status of Forest Conservation for Maintenance of Biodiversity in Nigeria, *in*: "Conservation of Plant Genes. DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

Gunn, G.R. and Dennis, J.V. 1979. "World Guide to Tropical Drift Seeds and Fruits." Quadrangle/The New York Times Book Co., New York, NY.

Hall, J. B. 1978. Sacred Groves. (mimeographed).

Hall, J.B. and Swaine, M.D. 1981. "Distribution and Ecology of Vascular Plants in a Tropical Rain Forest. Vegetation in Ghana." Dr. W. Junk Publication, The Hague, Netherlands.

Heywood, V.H. 1992. Efforts to Conserve Tropical Plants - A Global Perspective, *in*: "Conservation of Plant Genes. DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

IBPGR. 1990. "IBPGR Annual Report." FAO of UN, Rome, Italy.

Koagne, H. 1986. La dynamique des plantes et derives alimentaires dans la Chefferie Bafoussam. Memoire de Maitrise, Department of Geography, University of Yaounde, Cameroon (unpublished).

Lanzara, P. and Pizzettii, M. 1977. *in:* "Guide to Trees," S. Schuler, ed., Simon & Schuster, New York, NY.

Novak, F.A. 1966. "The Pictorial Encyclopedia of Plants and Flowers." Paul Hamlyn. London. Crown Publishers Inc., New York, NY.

Sanago, D. 1983. Bois sacre: temple ou ecole? Revue Ivoirienne d'Anthropologie et de Sociologie 3:69-92.

Taylor, C.J. 1962. "Tropical Forestry With Particular Reference to West Africa." Oxford University Press, London, UK.

UNESCO MAB. 1990. "Biosphere Reserves." UNESCO, Paris, France.

Appendix I. Legendary and Mythical Plants.

Adansonia digitata, Baobab
Balanites aegyptiaca, Desert Date or Soap Berry
Borassus aethiopum, Fan Palm
Ceiba pentandra, Silk Cotton Tree
Diospyros mespiliformis, West African Ebony Tree
Distemonanthus benthamianus, African Satinwood
Ficus species, Fig Trees
Gardenia ternifolia
Laurus nobilis, Laurel or Sweet Bay
Lodoicia maldivica, Coco-de-mer
Mandragora officinarum, Mandrake
Milicia (Chlorophora) excelsa, Iroko
M. regia, Iroko
Morus mesozygia
Myrtus communis, Myrtle
Okoubaka aubrevillei
Papax quinquefolium, Ginseng
P. schinseng, Ginseng
Punica granatum, Pomegranate
Quercus species, Oak
Solandra species
Tabernanthe iboga
Welwitchia mirabilis

Appendix II. Symbolic Plants.

Species	Symbol of
Adansonia digitata, Baobab	Fertility
Asparagus crispus	Fertility and prosperity
	domestic animals
Baphianitida, Camwood	Intelligence
Buxus sempervirens, Box	Palm Sunday
Cassytha filiformis, Dodder	Love
Cola acuminata, Commercial Cola Nut	Welcome and hospitality
C. nitida, Bitter Cola	Love and friendship
	Reconciliation and friendship
Commiphora africana, African Bdellium	Immortality
Costus afer, Ginger Lily	Purification from sin
Cuscuta australis, Dodder	Love
Dracaena arborea	Peace
Elaeis guineensis, Oil Palm (fronds)	Palm Sunday
Elettaria cardamomum, Cardamon	Hospitality
Erythrina crista-galli var. *variegata*	Evil
Ficus species (with abundant fruits)	Fertility
	Fecundity among Ando of Cote d'Ivoire
Gardenia species	Graceful charm
Ginkgo biloba, Ginkgo, Maidenhair Tree	Longevity
Hyssopus officinalis, Hyssop	Purification from sin
Iris florentina, Iris	Power and majesty
Kigelia africana, Sausage Tree	Fertility

Appendix II. Symbolic Plants. (Continued)

Species	Symbol of
Laurus mobilis, Sweet bay or Laurel	Victory
Marantochloa species	Maleness
Mussaenda erythrophylla, Ashanti Blood	War
Nigella damascena, Love-in-a-Mist	Love
Nymphaea lotus, Water Lily	Fertility
	Good or evil
	male or female
	light or darkness
	Immortality, purity and resurrection
	Peaceful intent
	Purification
	Regeneration
Olea europaea, Olive	Peace
O. europaea, Olive (the oil)	The Holy Spirit
Paeonia species, 'King of Flowers'	Wealth and honor
Papaver rhoeus, Poppy flower	Remembrance Day
Pinus species, Pine	Integrity and dignity
Portulaca oleracea, Purslane, Pigweed	Goodwill
	Peace
Prunus domestica, Plum	Physical charm and loveliness of women as well as of the spirit
P. persica, Peach	Longevity
Punica granatum, Pomegranate	Fertility
Rosa species, Rose	Love
Rosmarinus officinalis, Rosemary	Fidelity and remembrance
Salix woodii, Wild Willow	Palm Sunday
Thaumatococcus danielli, Katemfe	Female fertility
A tree in African myth and stories	Wisdom, authority and custom
	Mediator and judge
A tree in glazed containers	Those qualities that enrich life
Bamboo in Chinese mythology	Virtues such as purity of mind, fidelity, humility, wisdom, gentleness
	submission
Firewood	Culture, refinement and nobility
Orchids	of character

Appendix III. Religious Plants.

Traditional Religion

Adansonia digitata, Baobab
Alstonia boonei
Blighia sapida, Akee Apple
Ceiba pentandra, Silk Cotton Tree
Chrysophyllum albidum, White Star Apple
Detarium microcarpum, Tallow Tree
D. senegalense, Tallow Tree
Elaeis guineensis var. *idolatrica,* King Oil Palm
Ficus species, Fig Tree

Appendix III. Religious Plants.(Continued)

Traditional Religion (Continued)

Kigelia africana, Sausage Tree
Lodoicea maldivica, Coco-de-mer
Milicia (Chlorophora) excelsa, Iroko
M. regia, Iroko
Okoubaba aubrevillei
Pennisetum purpureum, Elephant Grass
Sarcostemma viminale
Sclerocarya caffra, Cider Tree
Tabernanthe iboga
Theobroma cacao, Cocoa

Islam

Cola acuminata, Commercial Cola Nut Tree
C. nitida, Bitter Cola
Dictamnus albus, Gas Plant
Gloriosa superba, Climbing Lily
Ixora grandifolia
Nelumbo nucifera, Lotus-flower
Nymphaea lotus, Water Lily
Sesamum indicum, Sesame or Beniseed
Thevetia peruviana, Exile Oil Plant

Other Religions

Amanita muscaria, Death Cap
Dianthus caryophyllus, Carnation
Nicotiana rustica, Wild Tobacco
Psilocybe mexicana
Verbena officinalis, Vervain
Viscum album, Mistletoe

Christ

Acanthus spinosus, Spiny Bear's Breach
Ailanthus altissima, Chinese Tree of Heaven
Anemone coronaria, De Caen Giant Flowered French or Blood Drops of Christ
Caladium bicolor, Heart of Jesus
Canotia species
Crataegus oxyacantha, Hawthorn
Euphorbia millii, Crown-of-Thorns or Christ's Thorn
Holacantha emoryi, Desert Crucifixion -thorn
Koeberlinia species
Palirus spina-christi, Christ's Thorn
Passiflora caerulea, Passion Flower
P. edulis, Passion Flower
P. glabra, Stinking Passion Flower
P. laurifolia, Water-lemon
P. quadrangularis, Giant Granadilla
Poterium spinosum, Spiny Burnet
Porana paniculata, Christ Plant or Christmas-Vine
Tragia species
Ziziphus spina-christi, Christ's Thorn

Appendix III. Religious Plants.(Continued)

The Virgin Mary

Anastatica hierochuntica, Resurrection Flower, Mary's Flower or Rose of Jericho
Lilium candida, Madonna Lily
Merremia discoidesperma, Mary's-bean or Crucifixion-bean
Silybum marianum, Blessed Mary Thistle

Biblical Plants

Boswellia carteri, Frankincense
B. papyrifera, Frankincense
B. sacra, Frankincense
Commiphora gileadensis, Balm of Gilead
C. molmol, Myrrh
C. myrrha, Myrrh
Crocus sativus, Saffron Crocus
Cyperus papyrus, Papyrus
Ficus crica, Common Fig
F. sycomorus, Sycamore
Juglans regia, Walnut
Laurus nobilis, Bay Tree
Morus nigra, Black Mulberry
Myrtus communis Myrtle
Nerium oleander, Oleander
Nymphaea lotus, Water Lily
Ornithogalum arabicum, Star of Bethlehem
O. narbonense, Star of Bethlehem
O. umbellatum, Star of Bethlehem
Platanum orientalis, Oriental Plane Tree
Prunus armenaica, Apricot
Punica granatum, Pomegranate
Rubus sanguineus, Bramble
Sterbergia lutea, 'Lilies of the field'

Temples (Plants used in building or planted around)

Cantua buxifolia
Caralluma dalzielii
Cedrus libani, Cedar of Lebanon
Cupressus sempervirens, Cypress
Dipterocarpus trinervis
Ficus religiosa, Pepul or Bo-Tree
Ginkgo biloba, Ginkgo or Maidenhair-Tree
Helianthus annuus, Sunflower
Illicium anisatum
Juniperus drupacea, Juniper
J. excelsa, Juniper
Michelia champaca
Nandina domestica, Heavenly Bamboo
Phoenix dactylifera, Date Palm
Pinus halepensis, Allepo Pine
Plumeria acutifolia var. *rubra,* Frangipani or Temple Flower
Santalum album, Sandalwood
Savastana odorata, Holy Grass
Styrax officinalis, Storax

Appendix III. Religious Plants (Continued)

Temples (Plants used in building or planted around) (Continued)
Tagetes lucida, Marigold
Thespesia populnea, Portia Tree

Appendix IV. National, State or Emblematic Plants.

Country or State	Species
Alabama	*Camellia* species, Camellia
Alaska	*Myosotis alpestris*, Alpine Forget-me-not
Albania	*Quercus coccifera*, Kermes Oak
Andorra	*Leotopodium alpinum*, Edelweiss
Argentina	*Erythrina crista-galli*, Ceibo or Coral-tree
Arizona	*Cereus giganteus*, Saguaro Cactus
Arkansas	*Pyrus malus*, Apple blossom
Ascension	*Zantedeschia aethiopica*, Calla
Australia	*Eucalyptus globulus*, Blue Gum
Austria	*Lilium* species, White Lily
Barbados	*Poinciana (Caesalpinia) pulcherrima*, Barbados-pride
Belgium	*Papaver rhoeas*, Red or Coca Poppy
Bermuda	*Sisyrinchium bermudiana*, Blue-eyed Grass
Bolivia	*Cantua buxifolia*, Inca Magic-flower or Khantua
Borneo	*Nipa fruticans*, Nipa Palm
Brazil	*Caesalpinia echinata*, Pau Brazil
Bulgaria	*Rosa* species, Red Rose
Burma	*Oryza sativa*, Rice
Caicos Island	*Melocactus communis*, Turk's-cap Cactus
California	*Eschscholzia californica*, California Poppy
Cambodia	*Oryza sativa*, Rice
Canada	*Acer platanoids*, Maple
Canary Islands	*Dracaena draco*, Dragon Tree
Chile	*Lapageria rosea*, Chilean Bell Flower
China	*Narcissus tazetta* var. *orientalis*, Chinese Sacred-lily
Colombia	*Cattleya trianaei*, Mayflower Orchid
Colorado	*Aquilegia caerulea*, Rocky Mountain Columbine
Congo, Republic of	*Adansonia digitata*, Baobab
Connecticut	*Kalmia latifolia*, Mountain Laurel
Costa Rica	*Cattleya skinneri*, Guarai Orchid
Cuba	*Roystonea regia*, Royal Palm
Czechoslovakia	*Dianthus caryophyllum*, Carnation
Delaware	*Prunus persica*, Peach
Denmark	*Fagus sylvatica*, Beech
Dominican Republic	*Swietenia mahogani*, Caoba Mahogany
Ecuador	*Theobroma cacao*, Cocoa
Egypt	*Nymphaea lotus*, Water Lily
El Salvador	*Yucca elephantipes*, Spanish-bayonet
England	*Rosa gallica*, Red Tudor Rose (*Quercus* species Oak)
Eritrea	*Zantedeschia* species, Calla
Ethiopia	*Coffea arabica*, Arabian Coffee
Finland	*Convallaria majalis*, European Lily-of-the-Valley
Florida	*Citrus sinensis*, Orange Blossom
France	*Iris pseudacorus*, Golden fleur de lis
Georgia	*Rosa sinica*, Cherokee Rose

Appendix IV. National, State or Emblematic Plants. (Continued)

Country or State	Species
Germany	*Centaurea cyanus*, Cornflower or Keiserblume
Greece	*Viola odorata*, Viola
Guatemala	*Lycaste skinneri*, White-nun Orchid
Guernsey Island	*Nerine sarniensis*, Guernsey-lily
Haiti	*Roystonea regia*, Royal Palm
Honduras	*Pinus rigida*, Pitch Pine
Hungary	*Tulipa* species, Tulip
Idaho	*Philadelphus lewisii*, Mock Orange
Illinois	*Viola pedata*, Bird's-foot Violet
India (star of)	*Nelumbo nucifera*, Lotus-flower
	Rosa species, Rose
	Two crossed Palm branches
(shield of)	*Cynodon dactylon*, Bermuda Grass
Indiana	*Paeonia* species, Peony
Indonesia	*Jasminum sambac*, Melati Jasmine
Iowa	*Rosa virginiana*, Wild Rose
Iran	*Rosa* species, Rose
Ireland	*Trifolium repens*, Irish Shamrock
Israel	*Vitis vinifera*, Grape Vine
Italy	*Pinus pinea*, Italian Stone Pine
Japan	*Chrysanthemum* species, Chrysanthemum
	Paulownia tomentosa (*imperalis*), Empress Tree
Kansas	*Helianthus annuus*, Sunflower
Kentucky	*Solidago* species, Goldenrod
Korea	*Magnolia* species, Magnolia Flower
Laos	*Ficus religiosa*, Bo-tree or Pepul
Latvia	*Chrysanthemum leucantheum*, Ox-eye Daisy
Lebanon	*Cedrus libani*, Cedar of Lebanon
Liberia	*Elaeis guineensis*, Oil Palm
Libya	*Punica granatum*, Pomegranate
Liechtenstein	*Lilium bulbiferum* var. *croceum*, Orange Lily
Lithuania	*Ruta graveolens*, Rue
Los Angeles	*Strelitzia reginae*, Bird of Paradise Flower
Louisiana	*Magnolia grandiflora*, Southern Magnolia
Luxembourg	*Rosa* species, Rose
Madagascar	*Ravenala madagascariensis*, Traveller's Palm
Maine	*Pinus strobus*, White pine
Malaysia	*Cocos nucifera*, Coconut Palm
Maryland	*Rudbeckia hirta*, Black-eyed Susan
Massachusetts	*Epigaea repens*, Mayflower
Mexico	*Euphorbia pulcherrima*, Poinsettia
Michigan	*Pyrus malus*, Apple Blossom
Minnesota	*Cypripedium* species, Lady's Slipper
Mississippi	*Magnolia grandiflora*, Southern Magnolia
Missouri	*Crataegus coccinea*, Hawthorn
Monaco	*Dianthus caryophyllus*, Carnation
Montana	*Lewisia rediviva*, Bittercup
Morocco	*Quercus suber*, Cork Oak
Nebraska	*Solidago serotina*, Goldenrod
Nepal	*Nelumbium nelumbo*, Blue Lotus
Netherlands	*Citrus sinensis*, Orange
Nevada	*Artemisia tridentata*, Sagebrush

Appendix IV. National, State or Emblematic Plants. (Continued)

Country or State	Species
New Hampshire	*Syringia vulgaris*, Lilac
New Jersey	*Viola* species, Violet
New Mexico	*Yucca* species, Yucca
New South Wales	*Telopea speciosissimum*, Waratah
New York	*Rosa* species, Rose
New Zealand	*Agathus australis*, Kauri-pine
Nicaragua	*Ceiba pentandra*, Silk Cotton Tree
North Carolina	*Cornus florida*, Dogwood
North Dakota	*Rosa arkansana*, Wild Prairie Rose
Northern Ireland	*Linum usitatissimum*, Flax
Norway	*Calluna vulgaris*, Heather
Ohio	*Dianthus* species, Carnation
Oklahoma	*Phoradendron flavescens*, Mistletoe
Oregon	*Berberis aquifolium*, Oregon Grape
Panama	*Sterculia apetala*, Panama-tree
Paraguay	*Brunfelsia hopeana*, Jasmine-of-the-Paraguay
Pennsylvania	*Kalmia latifolia*, Mountain Laurel
Peru	*Cantua buxifolia*, Inca Magic-flower
Philippines	*Jasminum sambac*, Sampagita Jasmine
Poland	*Viola tricolor*, Red Pansy
Polynesia	*Cocos nucifera*, Coconut Palm
Portugal	*Lavandula stoechas*, Lavender
Puerto Rico	*Delonix regia*, Flamboyante or Flame Tree
Rhode Island	*Viola* species, Violet
Rumania	*Rosa* species, Dog Rose
San Marino	*Cyclamen neopolitanum*, Cyclamen
San Salvador	*Yucca elephantipes*, Yucca
Saudi Arabia	*Phoenix dactylifera*, Date Palm
Saxony	*Reseda odorata*, Mignonette
Scotland	*Onopordum acanthium*, Scotch Thistle
South Africa	*Protea* species, Protea
South Carolina	*Gelsemium sempervirens*, Yellow Jessamine
South Dakota	*Pulsatilla hirsutissima*, American Pasque Flower
Spain	*Punica granatum*, Pomegranate
Sri Lanka	*Corypha umbracalifera*, Talipot Palm
Sudan	*Acacia* species, Acacia
Sweden	*Linnaea borealis*, Twin Flower
Switzerland	*Leontopodium alpinum*, Edelweiss
Syria	*Anemone caronaria*, Windflower
Tennessee	*Iris* species, Iris
Texas	*Lupinus subcarmosus*, Bluebonnet
Thailand	*Oryza sativa*, Rice
Tibet	*Eugenia jambos*, Rose-apple
Trinidad & Tobago	*Heliconia humilis*, Lobster-claw
Tunisia	*Acacia* species, Acacia
Turkey	*Tulipa* species, Tulip
Turk's Island	*Melocactus communis*, Turk's Island Cactus
United Kingdom	*Quercus* species, Oak
United Nations	*Olea europaea*, Olive
Uruguay	*Erythrina crista-galli*, Ceibo or Cockspur-coral
USSR	*Helianthus annuus*, Sunflower
Utah	*Calochortus nuttallii*, Sego Lily

Appendix IV. National, State or Emblematic Plants. (Continued)

Country or State	Species
Vatican City, State of	*Lilium longiflorum* var. *eximum*, Easter Lily
Venezuela	*Tabebuia pallida*, White-cedar
Vermont	*Trifolium pratense*, Red clover
Victoria	*Epacris impressa*, Common Heath
Virginia	*Cornus florida*, Flowering Dogwood
Wales	*Narcissus pseudo-narcissus*, Daffodil or Trumpet Narcissus
Washington	*Rhododendron californica*, Coast Rhododendron
West Africa	*Elaeis guineensis*, Oil Palm
West Indies	*Plumeria* species, Frangipani
West Virginia	*Rhododendron maximum*, Rhododendron
Wisconsin	*Viola* species, Violet
Wyoming	*Castilleja linarifolia*, Indian Paintbrush
Yemen	*Coffea arabica*, Arabian Coffee
Yugoslavia	*Convallaria majalis*, Lily-of-the-Valley

Appendix V. Other Plants Protected by Various Means.

Plants Not Used as Fuelwood

Annona chrysophylla, Wild Custard Apple
A. senegalensis var. *deltoides*
A. senegalensis var. *senegalensis*
Anthocleista kerstingii
A. nobilis
Combretum molle
Distemonanthus benthamianus, African Satinwood
Elaeis guineensis, Oil Palm (kernels)
Lophira alata, Red Ironwood Tree
Newbouldia laevis
Premna quadrifolia
Sterculia setigera

Plants Not Cut Down

Acacia mellifera var. *detinens*, Hookthorn
Adansonia disgitata, Baobab
Ceiba pentandra, Silk Cotton Tree
Dalbergiella welwitschii
Dialium guineense, Velvet Tamarind
Distemonanthus benthamianus, African Satinwood
Loesenera kalantha
Milicia (Chlorophora) excelsa, Iroko
M. regia, Iroko
Newbouldia laevis
Okoubaka aubrevillei
Salacia debilis
Sambucus nigra, Elder
Spiropetalum heterophyllum
Spirostachys africanum, African Sandalwood
Stereospermum kunthianum
Ziziphus mucronatum, Buffalo Thorn

DNA BANK DEPOSITORIES IN DEVELOPING COUNTRIES: THE WAY FORWARD IN SUB SAHARAN AFRICA

A.A. Oteng-Yeboah
Department of Botany, University of Ghana
Post Office Box 55, Legon, Ghana

SUMMARY

Current knowledge about the wild and domesticated biodiversity in developing countries is reviewed. Global conservation approaches are described, and issues and concerns in the creation and maintenance of DNA bank depositories in developing countries, particularly in Africa south of the Sahara, indicate that attention must be focused on institutional capacity building.

INTRODUCTION

The concept of a DNA-bank depository is used here to refer to the conservation effort of both the wild and domesticated biodiversity at the genetic (plus molecular), species and ecosystem levels. Wild biodiversity refers to systems that exist naturally in the wild, without any husbandry or human care. Domesticated biodiversity refers to systems that have become domesticated by man, and over the years have been selected and cultivated as crops for specific benefits to man. Domesticated biodiversity may be considered only at the genetic and species levels. Considerable knowledge is available about domesticated biodiversity; and the search for more information is continuing. The least known crop varieties in indigenous societies in the developing regions of the world are now receiving attention, and germplasm collections are being made, screened and documented.

The intensity in the search for these lesser known crop varieties comes in the wake of the search for genes that contribute to such heritable and preferred qualities as disease-resistance, pest-resistance, desirable food quality and environment-tolerant characteristics. Because of their proven importance to man, several conservation methods have been developed to ensure that domesticated biodiversity is not lost (Gopalan et al., 1986). There is a long history of plant gene conservation starting with early civilization through the periods of early European plant collecting expeditions, many of which later ended in the establishment of botanical gardens, arboreta and herbaria in strategic places in Europe, America and other colonial settlements in the tropics. The concept of Vavilov's centers of crop diversity has enabled botanists to identify the main origins of genetic resources and their trajectory of diffusion (Juma, 1989). These centers (Table 1) have not only been expanded to cover wider areas, but are now considered to be associated with areas in which plant major crop domestications occurred (Zhukovsky, 1975). The majority of these Vavilovian centers are located in the less developed countries of Africa, Asia and S. America. The evolution of the global gene banks is closely linked to plant collection activities either for taxonomic studies or breeding. Particularly for breeding purposes and to ensure that germ plasm collections can always be available in living form, the Consultative Group on International Agricultural Research (CGIAR) was set up in 1971 to support a global network of gene banks. These gene banks are located at 13 international agricultural research centers (IARCs) (Table 2). The International Board for Plant Genetic Resources (IBPGR)

Table 1: Vavilov Centers of Crop Diversity

Center	Location	Continent
1.	Mexico-Guatemala	Central America
2.	Peru-Ecuador-Bolivia	South America
3.	Southern Chile	South America
4.	Brazil-Paraguay	South America
5.	North America	North America
6.	Ethiopia-Kenya-Somalia	Africa
7.	Central Asia	Asia
8.	Mediterranean	Africa and Europe
9.	Indo-Burma	Asia
10.	Asia Minor	Asia
11.	Siam-Malaya-Java	Asia
12.	China	Asia

was set up by CGIAR in 1974 as an institution to deal with plant genetic resources to guarantee long-term operations, ensuring adequate and equal access to germ plasm globally in the search for High Response Varieties (HRVs). The IBPGR has full access to the knowledge and expertise built by FAO. It operates like a UN agency having full access to its member countries, but is not accountable to FAO. Thus, it is an independent body, primarily for the promotion of plant genetic resources.

The concept of genetic resources refers to the value found in the information contained in biological material either in the form of genes or chemicals for a particular function. Thus, it differs from the concept of biological resources for their physical attributes. For example, a maize seed is a biological resource if it is eaten, but it becomes a genetic resource if it is used as a source of a disease-resistant gene for breeding. Or a potion from a herb is a biological resource when consumed directly but it becomes a genetic resource when the active chemical is isolated and used as a model for a new drug. The impediments to germplasm conservation stem from ownership of the resource and the sharing of the proceeds accruing from the value attached to the biological material. This brings into focus aspects of Farmers (Indigenous Peoples) Rights (FR), Intellectual Property Rights (IPR), Plant Breeders Rights (PBR), and Biotechnology and its transfer, etc. These issues have been discussed at length in numerous international groups such as GATT (General Agreement on Tariffs and Trade); WIPO (World Intellectual Property Organization); ARIPO (African Intellectual Property Organization); UPOV (Union for the Protection of New Varieties of Plants); CGIAR; UNCED (United Nations Conference on Environment and Development); UNCTAD (United Nations Conference on Trade and Development); FAO; and in several research journals and reviews (Acharya, 1992; Cohen, 1992; Juma, 1989; Khalil et al., 1992; Reid, 1992).

Currently access by farmers in less developed countries to most genetic resources held in various gene banks is through plant breeders from commercial seed companies and these usually carry a very high price tag. Issues such as these are expected to be picked up at national, regional or international levels, in order to direct attention towards ensuring that farmers are provided with fair access to the genetic diversity which they have conserved over the centuries and which now have been refined as genetic

Table 2: CGIAR Supported Research Centers

Center	Year of Establishment	Location	Programs
IRRI	1960	Los Banos Philippines	Rice, Rice-based cropping systems
CIMMYT	1966	Mexico City Mexico	Maize, Bread wheat, Durum wheat, Barley, Triticale
IITA	1967	Ibadan, Nigeria	Farming systems, Maize, Rice, Sweet potato, Yams, Cassava, Cowpea, Lima bean, Soybean
CIAT	1968	Cali, Colombia	Cassava, Field beans, Rice, Tropical pastures
CIP	1971	Lima Peru	Potato
WARDA	1971	Monrovia Liberia	Rice
ICRISAT	1972	Hyderabad India	Chickpea, Pigeon pea, Pearl millet, Sorghum, Groundnut, Farming systems
ILRAD	1973	Nairobi Kenya	Trypanosomiasis, Theileriosis
IBPGR	1974	Rome Italy	Plant genetic resources
ILCA	1974	Addis Ababa Ethiopia	Livestock production systems
ILFRI	1975	Washington USA	Food policy
ICARDA	1976	Aleppo Syria	Farming systems, Wheat, Barley, Tricale, Lentil, Chickpea, Broadbean, Forage crops
ISNAR	1980	The Hague Netherlands	National agricultural research

resources through technology. The convention on biological diversity (UNEP, 1992) which was presented at the UN conference on Environment and Development in Rio de Janeiro, Brazil, in June 1992 has provisions for many of these issues raised above. Hopefully, when the document is ratified and pertinent articles are exhaustively discussed, there will be programs and actions to ensure a balanced world approach.

Knowledge about the wild biodiversity is varied, and particularly from the developing countries, this is very low. From a genetic viewpoint, very little is known about the cytological and inheritance information of tropical species. Such information as chromosome number, chromosome morphology and breeding behavior and systems continue to remain virtually unknown. At the species level, the knowledge of plant floristic diversity is at the exploratory (pioneer) and the consolidation (herbarium and to a small extent field information) phases (Davis and Heywood, 1963). According to Hepper (1979), there are only a few areas in Africa south of the Sahara whose flora are

moderately known floristically. Leonard (1975) attributes this poor knowledge to lack of explorations made into the flora of Africa south of the Sahara. The shortage of facilities (such as herbaria, libraries and botanic gardens) and trained staff (such as taxonomists) in most tropical countries are also considered to account for the low knowledge of the species biodiversity (Heywood, 1991; Oteng-Yeboah, 1993).

The level of knowledge at the ecosystem level follows the same pattern as for the genera and species. The knowledge is available mainly at the level of general plant formations such as forest, savanna, desert and various intergradations between these. Detailed knowledge and understanding of ecosystems functions and structural dynamics as well as the knowledge of the special uses of their species components possessed by the indigenous people of these areas in the tropics are lacking. Considering the alarming rates at which pristine tropical forests and habitats are destroyed (Guppy, 1984; WRI, 1988) coupled with the estimate that the world's tropical forests contain about half of the world's species (Wilson, 1988) and that even within this biome a disproportionate amount of the biodiversity is concentrated in a relatively small number of areas (Myers, 1988a,b), much of which are yet to be discovered through research and field expeditions before their uses are documented, it becomes a source of worry to observe the current low level of knowledge of wild biodiversity in less developed countries. The disagreement between experts on how best to achieve the objective of conserving the forests of Africa, and elsewhere in the tropics, is also worrying. One expert group believes that the rapid economic development being promoted by the World Bank and other aid agencies is likely to lead to (and has in fact led to) the opening up of forest areas, the intensive agricultural use of forest land, and unsustainable use of forest resources. Another expert group believes that the generation of wealth and employment and the alleviation of poverty, which are targets of developmental assistance, are essential if we are to create a social and economic climate in which forest conservation programs can have a chance of succeeding.

The revelation from this disagreement is that the Tropical Forest Action Plan (TFAP) devised by FAO, which is conceived as a plan to save the tropical forests, is now claimed by conservation and human rights organizations in both the developed and developing world, as actually accelerating forest loss and harming the interests of forest dwelling people (Sayer, 1992).

The recent Rio conference has brought a global awareness to issues of biodiversity, conservation and development, directly and indirectly underscoring the conservation programs and strategies already outlined by such international institutions as IUCN, WRI, WWF, ODA, UNEP, etc. for natural habitats all over the world.

DNA-BANK DEPOSITORIES

The DNA-bank depositories in both the developed and less developed countries are maintained under two main approaches, relating to the main objective envisaged in conservation. These approaches are *ex situ* and *in situ*. *Ex situ* approaches refer to the collection and maintenance (storage or otherwise) of parts or full individuals or their populations and communities in facilities *away* from their original sites or places where they normally grow. *In-situ* approaches refer to the maintenance of the biodiversity in the form of the individual species, populations of the individual or the entire community at the *same place* where their evolution has been a continuous process.

Ex-situ Conservation

This form of conservation is practiced either in the laboratory or in the field.

1. *Laboratory gene-bank conservation*

a. *Seed storage:* where seeds are collected, dried, sealed in airtight containers and stored at temperatures acceptable for base collections (-1° to -20°C), active collections (at 0° to 15°C) and working or breeders collections (ambient or air conditioned room). Seed storage is the common way of storing germplasm due to low cost and low tech equipment required (Krogstrup et al., 1992). Temperature is one of the main factors determining seed viability in storage, and quantitative relationship between seed longevity and temperature among several diverse plant species has been established (Dickie et al., 1990). According to Roberts (1973, 1975), there are two types of seeds depending on their physiological response to desiccation and low temperature, and these have been classified as "*orthodox*" and "*recalcitrant.*" The "*orthodox*" seeds are those that can be dried to low moisture contents and tolerate freezing temperatures. These seeds include much of the common arable crops (cereals, legumes etc.) and most wild species in the temperature zone.

The "*recalcitrant*" seeds die if their moisture content is reduced below some relatively high critical value (12 - 31%). Even if stored in a moist condition they lose their viability in a relatively short time from a few weeks to a few months. This type is common in the humid tropics with large seeds, which have no dormancy and which germinate almost at once (Hawkes, 1982). Cocoa, rubber, mango etc. are examples of such seeds that are difficult to store (Roberts et al., 1984).

b. *In vitro* environment is an artificial growth medium environment. The plant materials used are tissue or organ cultures of embryo, pre-existing axillary buds on stems or storage organs (meristem or shoot tips) and other somatic material which will yield adventitious buds or embryo in bud. This method is currently focused on target crops that have conservation problems either because they have recalcitrant seeds or because they are vegetatively propagated (IBPGR, 1984; Van Sloten and Reid, 1992).

c. *Cryopreservation.* This involves cryogenic storage which is based on the reduction and subsequent arrest of metabolic functions of biological material by imposition of ultra low temperature (-196°C = Liquid nitrogen) while maintaining viability (Kartha, 1985). When plant cells are exposed to -196°C cellular metabolic activities cease due to unavailability of liquid water. Genetic changes are minimized. Plant material for cryopreservation is obtained from seeds, embryos, shoot tips, apical meristems and pollen. Orthodox seeds can usually be cryopreserved provided the moisture content is adjusted to 5-10%, depending on the species (Stanwood, 1985). Somatic embryonic cell suspensions are considered the easiest plant material for cryopreservation (Winters, 1985). All other plant materials have associated problems. Cryopreservation appears to be a valuable means of long term storage (base collection) since the method provides physical stability and requires little management and low input of consumable material (Van Sloten & Reid, 1992).

Field Genebank Conservation

a. *Botanic gardens.* The living collections of the world's botanic gardens and arboreta represent the largest assemblage of plant diversity outside nature, running into tens of thousands of species (Heywood, 1992a). Most of the material exists as very small accessions of one or few individuals which will not represent an adequate sample

of the genetic variation of the species for a genetic resource collection (Heywood, 1992a).

Recognizing their activities as part of a wider global effort to preserve biological diversity, the more than 300 member botanic gardens in 67 countries have constituted a global network to encourage and coordinate the conservation activities. They now have general priorities which are intended to guide their activities. These priorities are of two parts for the conservation of wild and cultivated species (WWF - IUCN - BGCS, 1989; Heywood, 1992b).

For wild species, the five priorities given are:

rare and endangered species (at national, regional and global level)
economically important species, particularly medicinal plants (Heywood, 1991)
species required for restoration or rehabilitation of ecosystems
keystone species (known to be of particular significance in the maintenance and stability of ecosystems)
taxonomically isolated species.

For cultivated species, the two priorities given are:

primitive cultivars (land races)
semi-domesticated species

For cultivated species, botanic gardens are expected to complement the work covered by such agencies as FAO, CGIAR, etc., rather than duplicating it (Hoyt, 1988).

b. *Orchards*: for the conservation of various cultivars of fruit tree crops such as banana, oranges, avocados, guava, mangoes, bread fruit, etc.; and for vegetatively cultivated crops such as those with bulbs, corms, tubers and rhizomes like onion, sweet potato, cocoyam, yam, sugar cane, etc. This method is also suitable or convenient for plants with recalcitrant seeds and species that do not produce seeds.

c. *Arboreta:* like the botanic gardens, arboreta are for the conservation of tree crops for re-afforestation (plantation), ornamental, or special purpose.

d. *Private and amenity forest plantations:* trees cultivated, owned and managed by individuals, communities or institutions for the provision of shade, shelter, landscape beauty and in some cases to meet local fuel wood, pole and other wood requirements.

e. *Zoological gardens*: like the botanical gardens, but hold collections of animals.

In-situ Conservation

Much of *in situ* conservation are legally protected areas arranged by the IUCN (1986) in categories of ascending order of degree of direct human use permitted in the areas.

Category I is a scientific reserve/strict nature reserve for the exclusive protection of flora and fauna resources. Any conflicting activities or land uses like hunting, fishing, forestry, mining, etc., are prohibited.

Category II is a National Park in which commercial extractive uses are not permitted. It is a natural area, not materially altered by human activity.

Category III is a Natural Monument/Natural Landmark to protect and preserve nationally significant natural features because of this special interest or unique characteristics. These are usually small areas.

Category IV is a wildlife sanctuary or Managed Reserve for the protection of characteristic wildlife, especially migratory bird communities and of threatened animal or plant species.

Category V is Protected Landscapes and Seascapes to maintain nationally significant natural landscapes which are characteristic of the harmonious interaction of man and land while providing opportunities for public enjoyment through recreation and tourism.

Category VI is Resource Reserve to protect natural resources of the area for future use and prevent or contain development activities that could affect the resource pending the establishment of objectives which are based upon appropriate knowledge and planning.

Category VII is Anthropological Reserve/Natural Biotic Area to allow the way of life of societies living in harmony with the environment to continue undisturbed by modern technology.

Category VIII is Game Production Reserve for the conservation, management and propagation of wildlife and its habitat.

There are other management Categories such as Biosphere Reserve, World Heritage sites and Ramsar Wetland.

Biosphere Reserve (Category IX) is to conserve for present and future use the diversity and integrity of biotic communities of plants and animals with natural ecosystems, and to safeguard the genetic diversity of species on which their continuing evolution depends. Internationally designated site by UNESCO managed for research, education and training.

World Heritage Site (Category X) is to protect the natural features for which the area is considered to be of outstanding universal significance.

Ramsar Wetland (Category R) is a wetland that is of international importance in terms of ecology, botany, zoology, limnology or hydrology as a water fowl habitat.

At the indigenous communities level in Africa and Asia, one form of vegetation protection is the concept of Sacred or Traditional groves. The legislation on these groves is based on local bye-laws, customs, rites and taboos. These groves are extant and flourishing because of local involvement in the conservation effort.

ISSUES AND CONCERNS

The panoramic impression created in the above review of issues related to DNA-bank depositories brings up concerns for less developed countries, particularly those in Africa south of the Sahara.

1. *Institutional capacities*

The effectiveness of any conservation approach, either *ex situ* or *in situ*, depends on the capacity of the relevant institution to manage it. Infrastructural requirements, research and development outlook, and institutional facilities and manpower availability are such capacities.

(a) A number of infrastructural requirements have to be in place for every aspect of the mechanism of conserving and the security of the material which is to be/has been conserved. For appropriate *ex situ* laboratory genebank conservation, there is the requirement, primarily, of temperature and moisture control systems. These come under equipment procurement and the generation of power (electricity) to enable this equipment to function.

The availability and continued maintenance of this equipment are issues of

technology transfer. Being usually imported items, the frequency of breakdown and lack of spare parts and/or trained personnel to fit these, make the conservation effort worthless. Similarly, electricity generation and its attendant problems of cost and maintenance reduce the incentives for *ex situ* laboratory gene bank conservation.

For *ex situ* field gene bank conservation, the concern is in the direction of programs. The engagement and promotion of horticultural management of exotics at the expense of indigenous plants, for sale, landscaping etc. appear to be high on the priorities of most botanical gardens in the tropics.

Obviously, there is need for trained personnel to take advantage of the new challenges based on the WWF- IUCN-BGCS (1989) list of priorities. There is also the need for the establishment of many more *ex situ* field genebanks in different ecological zones to take care of indigenous plant material.

For appropriate *in situ* conservation, the targeted management regime by the concerned institution must be seen to function through adequate funding and legal enforcements. Sequel to logistic problems and lack of monitoring equipment and personnel, many of the protected areas have become very highly vulnerable to poaching and illegal and/or unauthorized land uses.

(b) The other requirement for institutional capacity is in the area of research and development (R and D) of the material for conservation. This sphere is quite successfully utilized in the CGIAR-sponsored research stations, but to a small extent in national institutes. Research and development capacity is the only way through which national biological resources would be identified locally and converted into valuable genetic resources, thus ensuring the development of a local technology which can be maintained.

(c) Shortage of such institutional facilities as herbaria, libraries and botanic gardens, and general lack of taxonomic practitioners in less developed countries is a major blow in the advancement of floristic knowledge. With fewer or no floristic explorations currently going on, and the absence of an urgency to train taxonomists in the tropical countries, there is a race against time to ensure that potential new plant discoveries in the protected areas remain intact until they can be identified, documented, established, and adequately protected.

2. *Search for new varieties*

There are risks in the quest to grow more food, to feed the increasing populations in the tropics, by using modern high yielding varieties (HYVs). The risks involve the displacement of traditional crops resulting in genetic erosion, and the dying out of the knowledge of how to use traditional crops even before the plants themselves disappear (Anon., 1993). Members of traditional communities, particularly in Africa, have unique understanding and interpretation of their surroundings, including an understanding of the crops and weeds that are used for food and medicine and their relationship to the environment.

The search for new crop varieties therefore requires a close collaboration and cooperation between the traditional farmers, and national research institutions. The ethnobotanical research component of plant science institutions is a very vital contribution toward the search for new varieties.

3. *International co-operation*

Recognizing current difficulties in arbitrating on Farmers Rights (FR). Intellectual Property Rights (IPR), Plant Breeders Rights (PBR), Technology and Biotechnology transfers and sovereignty over genetic resources, emphasis is expected to

be focused on negotiations for mutual co-operative programs, transfers of financial and/or technical resources and new property regimes between the developed and less developed countries in order to promote investments in conservation and research bringing up appropriate balance between centralized research and local adaptation (Reid, 1992). Provisions of the Convention of Biological Diversity seeks to promote this, and it should be supported.

The intransigence of developed countries' negotiating positions for increased and uniform IPR protection, increased conservation and continued free access to unimproved genetic material, and that of the less developed countries' position against strengthened/uniform IPR protection and for increased sovereignty over resources, recognition of farmers' rights and matching technology transfer with increased conservation investment, should now be seen to be fading out.

CONCLUSION

In the foregoing, a review of current knowledge about the wild and domesticated biodiversity and the approaches to their conservation has been made. The options open to developing countries in the creation and maintenance of DNA-bank depositories do not appear to be wide. This is because of the poor state in which the economies of these countries are found. The options should therefore be those that will take into account such limitations as lack of skilled personnel and equipment to enable the establishment of a national capacity for genetic resources conservation.

Skilled personnel in all aspects of conservation efforts from the basic research scientists like ethnobotanists, taxonomists through applied scientists like plant breeders and genetic engineers, agronomists and tissue culture specialists, to technicians like refrigeration specialists need to be trained. For some strange reason of lack of job outlets available to taxonomy students, this field has remained unattractive to students in the third world (Oteng-Yeboah, 1991). Efforts need to be made to ensure extensive interest and specialization in the study of taxonomic botany in the tertiary institutions of the third world in order to remove the taxonomic impediment (Oteng-Yeboah, 1993). Job outlets should be found for such specialists.

At the local scene, parataxonomists should be identified and trained to initially act as the vanguard of the wild biodiversity that has not been studied scientifically. The choice and use of equipment must be expertly advised and handled, taking into consideration the local availability of maintenance. Sequel to current conservation imperatives, there is need to undertake a detailed review of existing legislation in order to identify loopholes and new needs for consideration and redress. With the increased capacity to screen plants for a wide range of uses, the scope for potentially useful plants is expanding, and national legislation should therefore reflect this change. Legislation on conservation should not now be concentrated on the perceived economic or ecological benefit of a plant. All plants are now to be considered potentially useful, and legislation should reflect that.

Strategies for screening plants, including the objectives and methodologies, should include recognition of and collaboration with local personnel to enhance capacity building. The contracting screening agencies must be made to enter into agreements to train local personnel in biochemical and chemical extracting methods and in assaying techniques, in addition to the sharing of dividends in the produce developed after the screening.

There is need to establish a framework within government organization (GO) in

which the role of community based groups such as non-governmental organizations (NGOs) will be recognized. The NGOs act as bridges with farmers, building on local knowledge and resources and providing them with fair access to the genetic diversity which they have conserved over the centuries. Through the establishment of NGO-GO framework in which the informal and formal sectors can work more closely together for their mutual benefit, the prospect for conservation of plant material will be enhanced and also the conservation of indigenous knowledge of how to grow and use that plant material will be enhanced. This ensures that the local communities benefit from modern breeding techniques without losing control and access over their material. The framework should allow for experimentation and flexibility so that local communities can establish their own methods of conservation within their labor and material limitations.

The Plant Genetic Resources Center of Ethiopia (PGRC/E) and the Environment and Development Action (ENDA) of Zimbabwe are leading NGOs in Africa who are effectively using this framework between themselves, the government and the farmers.

The field of biotechnology is rapidly expanding and governments are expected to take the initiative to ensure the possible use of biotechnological methods in the conservation of plant species in their areas which are close to extinction. This initiative is to be taken to engage available scientists in identifiable national research programs so as to forestall their attention being drawn into the research programs that reflect the agenda of developed countries. Many of the programs supported in the third world countries for the maintenance of biological diversity by the industrialized countries, as a matter of fact, have the goals and objectives of the sponsors (OTA, 1987).

REFERENCES

Acharya, R. 1992. "Intellectual Property, Biotechnology and Trade: the Impact of the Uruguay Round on Biodiversity. " Biopolicy International Series 4. ACTS Press, Nairobi.

Anonymous. 1993. Africa's gene pool: sink or swim? Spore Bi-monthly (February) No. 43, p.5.

Cohen, J.I. 1992. "Conservation and Use of Agro-Ecological Diversity." Biopolicy International Series 3. ACTS Press, Nairobi.

Davis, P.H. and Heywood, V.H. 1963. "Principles of Angiosperm Taxonomy." Oliver and Boyd, Edinburgh.

Dickie, J.B., Ellis, R.H., Kraak, H.L., Ryder, K. and Tompsett, P.B. 1990. Temperature and seed storage longevity. Ann. Bot. 65:197-204.

Gopalan, H.N.B., Karanja, W.K. and Taylor, A.R.D. 1986. The status of germplasm conservation: a review. Kenya Journal of Science and Technology, (B) 7(1):5-16.

Guppy, N. 1984. Tropical deforestation: a global view. Foreign Affairs 62(4):928-965.

Hawkes, J.G. 1982. Genetic conservation of "recalcitrant species"- an overview, *in:* "Crop Genetic Resources, The Conservation of Difficult Material," L.A. Withers and J.T. Williams, eds., International Union of Biological Studies Serie B42, Paris.

Hepper, F.N. 1979. Africa, *in:* "Systematic Botany, Plant Utilization and Biosphere Conservation", I. Hedberg, ed., Almqvist & Wiksell International, Stockholm.

Heywood, V.H. 1991. Botanic gardens and the conservation of medicinal plants, *in:* "Conservation of Medicinal Plants," O. Akerele, V.H. Heywood and H. Synge, eds., Cambridge University Press, Cambridge.

Heywood, V.H. 1992a. Efforts to conserve tropical plants -a global perspective, *in*: "Conservation of Plant Genes, DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

Heywood, V.H. 1992b. Botanic gardens and conservation: new perspectives, *in: "Ex situ* Conservation in Botanical Gardens," O. Hamann, ed., Opera Botanica 113:9-13.

Hoyt, E. 1988. Conserving the wild relatives of crops. IBPGR-IUCN-WWF, Rome and Gland.

IBPGR. 1984. "The Potential for Using *in vitro* Techniques for Collecting Germplasm." International Board for Plant Genetic Resources, Rome.

IUCN. 1986. "Managing Protected Areas in the Tropics" International Union for Conservation of Nature and Natural Resources, Gland.

Juma, C. 1989 "The Gene Hunters: Biotechnology and the Scramble for Seeds." Zed Books, London and Princeton University Press, Princeton.

Kartha, K.K. 1985. Meristem culture and germplasm preservation, *in:* "Cryopreservation of Plant Cells and Organs," K.K. Kartha, ed., CRC Press, Boca Raton, Florida.

Khalil, M.H., Reid, W.V. and Juma, C. 1992. "Property Rights, Biotechnology and Genetic Resources." Biopolicy International Series 7, ACTS Press, Nairobi.

Krogstrup, P., Baldursson, S. and Norgaard, J.V. 1992. *Ex situ* genetic conservation by use of tissue culture, *in: "Ex situ* Conservation in Botanical Gardens," O. Hamann, ed., Opera Botanica 113:49-53.

Leonard, J. 1975. Statistiques de progrès accomplis en 21 ans dans la connaissance de la flore phanerogamique africaine et malgache (1953-1973). Boissiera 24:15- 19.

Myers, N. 1988a. Threatened biotas: "hot spots" in tropical forests. The Environmentalist 8(3):1-20.

Myers, N. 1988b. Tropical forests and their species. Going, going.....? *in:* "Biodiversity." E.O. Wilson and F.M. Peter, eds., National Academy Press, Washington, D.C..

OTA. 1987. "Technologies to Maintain Biological Diversity." Office of Technology Assessment, Congress of the US, Washington, D.C.

Oteng-Yeboah, A.A. 1991. "Plant Taxonomy - a Neglected Science in the Third World," Ghana Science Association 17th Biennial Conference, UST Kumasi, Ghana.

Oteng-Yeboah, A.A. 1993. "Strategies towards Increased Knowledge of the African Biodiversity," International Conference on the Convention on Biological Diversity: National Interests and Global Imperatives, Nairobi, Jan. 26-29.

Reid, W.V. 1992. "Genetic Resources and Sustainable Agriculture: Creating Incentives for Local Innovation and Adaptation." Biopolicy International Series 2, CTS Press, Nairobi.

Roberts, E.H. 1973. Predicting the storage life of seeds. Seed Science Technology 1: 499-514.

Roberts, E.H. 1975. Problems of long-term storage of seeds and pollen for genetic resources conservation, *in*: "Crop Genetic Resources for Today and Tomorrow." O.H. Frankel and J.G. Hawkes, eds., Cambridge University Press, Cambridge.

Roberts, E.H., King, M.W. and Ellis, R.H. 1984. Recalcitrant seeds, their recognition and storage, *in*: "Crop Genetic Resources: Conservation and Evaluation," J.H.W. Holden and J.T. Williams, eds., Allen and Unwin, London.

Sayer, J.A. 1992 Development assistance strategies to conserve Africa's rainforests, *in:* "Conservation of West and Central African Rainforests," K. Cleaver et al., eds., World Bank Environment Paper No. 1, Washington, D.C.

Stanwood, P.C. 1985. Cryopreservation of seed germplasm for genetic conservation, *in*: "Cryopreservation of Plant Cells and Organs," K.K. Kartha, ed., CRC Press, Boca Raton, Florida.

UNEP. 1992. "The Convention on Biological Diversity," United Nations Environment Programme, Nairobi.

Van Sloten, D.H. and Reid, R. 1992. IBPGR's experience in *ex situ* conservation and the possible use of botanic gardens, *in: "Ex situ* Conservation in Botanical Gardens," O. Hamann, ed., Opera Botanica 113:19-23.

Wilson, E.O. 1988. The current state of biological diversity, *in*: "Biodiversity," E.O. Wilson and F.M. Peter, eds., National Academy Press, Washington, D.C.

Winters, L.A. 1985. Cryopreservation of cultured plant cells and protoplasts, *in:* "Cryopreservation of Plant Cells and Organs," K.K. Kartha, ed., CRC Press, Boca Raton, Florida.

WRI. 1988. "World Resources." World Resources Institute, Washington, D.C.

WWF-IUCN-BGCS 1989. "The Botanic Gardens Conservation Strategy." WWF-IUCN-BGCS, Gland and Richmond.

Zhukovsky, P.M. 1975. "World Gene Pool of Plants for Breeding: Mega-gene Centres and Micro-gene Centres." USSR Academy of Sciences, Leningrad.

NIGERIAN MEDICINAL PLANTS AND DANGER OF EXTINCTION

Zac O. Gbile
Ogun State University
Ago-Iwoye, Nigeria

SUMMARY

The history of botanical collecting in Nigeria is briefly reviewed. The early collectors, who were mostly medical practitioners, were not only interested in the collecting but also in traditional medicinal uses. Collection without propagation was the vogue; thus some of the species became extinct.

The methods of study devised by the ethnobotanical team sponsored by the Organization of Africa Unity are also discussed. The results are reflected in Appendix I and those of other surveys in Appendices II-IV. Species which are commonly found in herb markets, may face the danger of extinction if adequate measures are not taken because of their removal from the wild. Various conservation measures recommended include: establishment of patches or small enclaves with forests which are designed as juju shrines where traditional rites are performed; protection of individual plants; appeals to every local government in Nigeria for participation in the establishment of strict natural reserves; and encouragement of herbsellers to restrict their collection to unreserved areas.

INTRODUCTION

The awareness of therapeutic uses of plants is universal. Forests according to Maria Treben (1986) are God's own pharmacy. Forest species offer many utilitarian benefits of immediate use to mankind, one of the more important being medicinal plants on which about 80% of the population in Nigeria depend.

The collection of medicinal plants dates back to the 19th century when the first collections were made in the Northern and Southern Provinces of Nigeria by early explorers. The earliest collection from the Southern Provinces of Nigeria was believed to be one made by J. Kirkwood, a surgeon who sent plants to Potiver from old Cablabar about 1698 (Hutchinsen & Dalziel, 1954).

E. G. Irving ,who was also a surgeon, collected at Abeokuta. The genus *Irvingia* Hook f. was named after him. The Chief Medical Officer, Sir Gilbert Carter, in the company of J. W. Rowland, Botanist, and Dawodu, an African Assistant Curator (1899), also collected around Lagos and adjoining Yoruba country. Between 1854 and 1859, Dr. Baikie, a surgeon and naturalist, also collected around Lokoja.

The medical practitioners were not only interested in the collecting but also in the traditional medicinal uses of the plants. This is not only true of early explorers but it is true with Nigerian pharmacognosists, chemists, phytochemists, biochemists and botanists.

It has to be understood, however, that the plants collected by both old and new collectors were never replaced. It is not surprising that some old collections found in the Kew Herbarium cannot be traced back to any of the herbaria in Nigeria as a result of extinction.

METHODS

In the past, the knowledge of herbal medicine was usually passed from the head of the family to his descendants, mostly the beloved ones among them. As a result, secrecy dominated the practice of herbalism thus making it difficult for researchers to obtain useful information from traditional healers.

Realizing this impediment, the team of scientists sponsored by the Organization of African Unity to conduct an ethnobotanical survey of Western Nigeria between July and August, 1989 adopted a new strategy (Gbile, 1987). The author of this paper was a member of the team. The team, made up of doctors, pharmacists, botanists and traditional healers were divided into three groups. Group 1 was responsible for surveying herb sellers, market places and visiting all markets where medicinal plants and other medicinal compounds were sold. Group 2 took surveys in towns from traditional clinics, health centers and places of worship, while Group 3 was in charge of surveying villages and the countryside and evaluation of the flora and ecology of the natural environment. Each group was provided with funds to acquire plants and the various ingredients utilized. The informants were also compensated. A common format was designed to be used by all investigators. Information included the addresses of the healers, the age and sex, the vernacular and the scientific names of plants purchased, the parts used, diseases treated, pharmaceutical preparation, and route of administration. A form was completed for each recipe in a prescription. Before recipes were paid for, the herb sellers were implored to give the vernacular names of items sold. These were later identified by the botanists on the team (Gbile, 1989).

In similar manner, the herbarium staff of the Forestry Research Institute of Nigeria, Ibadan, carried out a similar survey in Southeastern Nigeria. In 1990 and 1992 the author undertook an ethnobotanical survey of the arid regions in Nigeria (Sokoto, Kaduna, Katsina, Kano, Bauchi and Borno) and Northeastern states (Bauchi, Yobe, Bornu, Adamawa and Taraba) respectively.

RESULTS

Appendices I-III show the commonly exploited medicinal plants in each subregion. Medicinal plants observed to be planted or protected in villages in Southeastern Nigeria are shown in Appendix IV. The cultivation (*ex situ*) is practiced on a very small scale by the traditional healers who use the plants. The different species are either planted in a single or double row on both sides of the road or path leading to the herbalists compound or the plants are grown in a plot where they grow to form a dense bush or juju shrine. Some useful species are protected in the farm lands.

DISCUSSION AND RECOMMENDATIONS

Plant material sold in herb markets is usually harvested from the wild, sales of which make plant recipes readily available as drugs. It is also a source of livelihood for the seller.

The main disadvantages result from the uncontrolled method of harvesting. As a result, many of the commonly used medicinal plants may be eliminated from our flora if nothing is done to check the methods of destructive exploitation. Plant species shown in Appendices I-III are some of the species commonly found in herb markets that are seriously facing the danger of extinction as a result of constant harvesting without replacement.

Tropical moist forests constitute the richest ecological region on earth. It is not unrealistic to suppose that within the foreseeable future, the fundamental modification of tropical moist forests could lead to the disappearance of as many as 1 million species (Gbile, 1987). In fact, it is likely that right now, at least one species is disappearing in these forests each day. Before many years go by, we could face a situation where one species becomes extinct each hour.

Steinlin (1982) estimated the global rate of tropical forest loss between 1981 and 1985 to be 0.8% annually and that of West African forests to be 4-6%. In Nigeria, the rate of deforestation of closed high forests for the same period is estimated to be 260,000 hectares per year for forests outside the reserves and 25,000 hectares per year for reserved forests.

Generally in the arid zone in Nigeria, especially where desert is rapidly encroaching, most plants face the danger of extinction. During the author's visit to Sokoto, where desert seriously encroaches, out of the 21 families representing the 63 plant species randomly collected from different sites, Leguminosae ranks highest, represented by 21 species; Graminae was second, represented by 7; Combretaceae and Meliaceae were third, represented by 4 species each; while Euphorbiaceae was fourth, represented by 3 members. All others are either represented by 1 or 2 species. From the data, it could be suggested that the members in the Leguminosae are more successful indigenous species in the desert-encroached areas and are probably more drought-resistant than other families represented.

Various conservation measures have previously been discussed (Gbile, 1992). In addition, establishment of patches or small enclaves within forests which are designed as juju shrines (where traditional rites are performed) is recommended. Individual plants may also be protected because of their edible and/or medicinal parts. Species of trees could also be preserved for provision of shade or medicinal purposes.

A serious appeal must be made to every local government in Nigeria for participation in the establishment of strict natural reserves where vegetation can be protected. Herb sellers should be confined to unreserved areas for the collection of their plant material and they should be fully involved in conservation for the day will come when most of the species commonly harvested as medicinal plants will disappear if adequate preventive measures are not taken. Attempts should also be made by traditional healers to establish medicinal plant gardens (Appendix IV).

REFERENCES

Gbile, Z.O. 1987. Forestry, Recreation and Health. The role of forestry in a depressed economy. Proceedings of the 17th Annual Conference of Forestry Association of Nigeria. A.B. Oguntala, ed.

Gbile, Z.O. 1989. Medicinal plant research in Western Nigeria. Forest resources development and the sources of local raw materials in Nigeria. J.A. Okojie and P.C. Obiaga, eds.

Gbile, Z.O. 1992. Status of forest conservation for maintenance of biodiversity in Nigeria. *in:* "Conservation of Plant Genes. DNA Banking and *in vitro* Biotechnology," R.P. Adams & J.E. Adams, eds., Academic Press, San Diego, CA.

Gbile Z.O. and Onyeachusim, H.D. 1990. Strategy for exploitation and conservation of medicinal plants. Paper presented at the symposium on the conservation of plant genetic resources. IITA Ibadan.

Hutchinson, J. and Dalziel, J.M. 1954. "Flora of West Tropical Africa." Crown Agents for Overseas Government and Administrations, Millbank, London.

Steinlin, H.J. 1982. Monitoring the tropical forest. The first step. UNASYLVA 34(137):2-4.

Treben, Maria. 1986. "Health through God's pharmacy. Advice and experiences with medicinal plants." Wilkeln Ennasthales, Steys, Austria.

Appendix I. Endangered Medicinal Plants in Southwestern Nigeria. Source: Ethnobotanical survey data of Southwestern Nigeria (1989).

ACANTHACEAE

Blepharis maderaspatensis (Linn.) Heyne ex Roth — *Brillantaisia patula* T. Anders.
Dicliptera alternans Lindau

AMARANTHACEAE

Puplia lappaceae Linn.

AMARYLLIDACEAE

Crinum jagus (Thoms.) Dandy — *Pancratium hirtum* A. Chev.

ANNONACEAE

Enania chlorantha Oliv. — *Monodora myristica* (Gaertn.) Dunal
Monodora tenuifolia Benth. — *Uvaria chamae* - P. Beauv.
Xylopia aethiopica (Dunal) A. Rich.

APOCYNACEAE

Alstonia boonei De Wilde — *Picralima nitida* (Stapf.) Th. et H. Dur.
Rauvolfia vomitoria Afz.

ARISTOLOCHIACEAE

Aristolochia albida Duchartre — *Aristolochia ringens* Vahl.

ASCLEPIADACEAE

Pergularia daemia (Forsk.) Chiov. — *Secamone afzelii* (Schult.) K. Schum.
Thylophora glauca Bullock

ASTERACEAE

Chrysanthellum americanum (Linn.) Vatke — *Crassocephalum rubens* (Juss. ex Jacq.) S. Moore
Eclipta prostrata (Linn.) Linn.

BALANOPHORANCEAE

Thonningia sanguinea - Vahl.

BIGNONIACEAE

Kigelia africana (Lam.) Benth. — *Newbouldia laevis* (P. Beauv.) Seeman ex Bureau

BOMBACACEAE

Bombax buonopozense P. Beauv.

BORAGINACEAE

Ehretia cymosa Thonning. — *Heliotropium indicum* Linn.

BRASSICACEAE

Rorippa madagascariensis (DC.) Hara.

BURSERACEAE

Canarium schweinfurthii Engl. — *Commiphora africana* (A. Rich.) Engl.

CAESALPINIACEAE

Erythrophleum guineense G. Don. — *Erythrophleum ivorense* A. Chev.

CHENOPODIACEAE

Chenopodium ambrosiodes Linn.

CLUSIACEAE

Carcinia kola Heckel.

COMBRETACEAE

Anogeissus leiocarpus (DC.) Guill. et Perr. — *Terminialia glaucescens* Planch. ex Benth.

Appendix I (Continued)

CUCURBITACEAE	
Lagenaria breviflora (Benth.) Roberty	*Momordica charantia* Linn.
EUPHORBIACEAE	
Croton penduliflorus Hutch.	*Croton zambesicus* Mill. Arg.
HYPERICACEAE	
Harungana madagascariensis Lam. ex Poir.	*Visimia guineensis* (Linn.) Choisy.
HYPOXIDACEAE	
Cucurligo pilosa (Schum. et Thonn.) Engl.	
ICACINACEAE	
Icacina trichantha Oliv.	
IRIDACEAE	
Gladiolus psittacinus Hook.	
MELIACEAE	
Khaya grandifoliola C. DC.	*Khaya senegalensis* (Desv.) A. Juss.
MENISPERMACEAE	
Chasmanthera dependens Hochst.	*Cissampelos mucronata* A. Rich.
Cissampelos owariensis P. Beauv.	*Sphenocentrum jollyanum* Pierre.
MIMOSACEAE	
Acacia nilotica (Guill. et Perr.) O. Ktze.	*Tetrapleura tetraptera* (Schum. et Thonn.) Taub.
MYRISTICACEAE	
*Pycnanthus angolensis (*Nelw.) Warb.	
MYRACEAE	
Eugenia aromatica (Linn.) Baill.	
OLACACEAE	
Olax subscorpioidea Oliver	*Ongokea gore* (Hua) Pierre
OLEACEAE	
Schrebera arborea A. Chev.	
OXALIDACEAE	
Oxalis corniculata Linn.	
PAPILIONACEAE	
Abrus precatorius Linn.	*Baphia nitida* Lodd.
Lonchocarpus cyanescens (Schum. et Thonn.) Benth.	*Pterocarpus osun* Craib.
PLUMBAGINACEAE	
Plumbago zeylanica Linn.	
POACEAE	
Cymbopogon citratus (DC.) Stap.	
POLYGALACEAE	
Carpolobia lutea G. Don.	*Securidaca longepedunculata* Fres.
RUBIACEAE	
Chassalia kolly (Schum.) Hepper	*Morinda lucida* Benth.
Schumanniophyton magnificum (K. Schum.) Harms	
VERBENACEAE	
Lippia multiflora Moldenke	
VIOLACEAE	
Hybanthus enneaspermus (Linn.) F.V. Nuell.	
VITACEAE	
Cissus populnea Guill. et Perr.	*Cissus quandrangularis* Linn.
ZINGIBERACEAE	
Aframomum melegueta K. Schum.	*Costus afer* Ker-Cawl.
Curcuma domestica Valeton	*Zingiber officinale* Rosc.

Appendix II. Medicinal Plants Sold in Herb Markets in Southeastern Nigeria. Markets at: Umuahia (U); Owerri (Ow); Orlu (OL); Enugu (E)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Adansonia digitata L. (Bombacaceae)	Ajii	B		Bark: Malaria, diaphoretic, enteritis
Aframomum melegueta K. Schum. (Zingiberaceae)	Osei Oseoji Okpai Ose oru	Fr. Fr. Fr. Fr. + + + + Sd Sd Sd Sd	Spice stimulant Ingredients in many native medicine preparations (e.g. for rashes itching etc.)	Spice; chiefly used in veterinary medicine and for flavoring cordials, spirits and wine
Aframomum sp. (Zingiberaceae)	Ose-oji olonire	Fr. + wsd	If the sweet pulp is put in the mouth when making request, one's request is sure to be granted	
Alstonia bonnei De Wild. (Apocynaceae)	Egu	B B	Gonorrhea, Malaria Fever	Bark: Gonorrhea, rheumatism, malaria and as antidote
Amphimas pterocarpoides Harms (Leguminosae)	Awo			Bark decoction used for treatment of dysentery
Aristolochia (planch) ringens Vahl (Aristolochiaceae)	Aka-ogwu	R R R R	Remedy for worm and pile	Root of species, *A. albida* as stomachic & for healing Guinea worm and syphilis
Austranella congolensis (DeWild)) A. Chev. (Sapotaceae)	Ekpiri	Sd		

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Baillonela toxisperma Pierre (Sapotaceae)	Annu-nu-eba Anara-okwe	Sd Sd Sd Sd	Employed against witchcraft	
Baphia nitida Lodd. (Leguminosae)	Abosi, uhies	Wd Wd Wd Wd	Wood pomadei used as base material for skin diseases	Inflammation, skin infections
Buchholzia coriacea Engl. (Capparidaceae)	Uuke; (Wandaful-Kola)	Sd	Gonorrhea, stomach problems, control of pregnancy. Seed eaten as cola-nut after boiling	Seed used in skin eruptions, for worms & pains; crushed seed pasted over stomach for difficulty in child birth
Caesalpinia bonduc (L.) Rooch. (Leguminosae)		Sd Sd Sd Sd	Used to protect children against ring-worm and other skin diseases	Leaves applied for and also given for worms in children
Ceiba pentandra (L.) Gaertn. (Bombacaceae)	Apu, Akpu Akwu	B	unknown	(Bark) stimulant; laxative, mouth wash; swollen fingers in enemas
Chasmanthera dependens Hochst. (Menispermaceae)	Mgburu-ogwu juruala	S	Gonorrhea	(Root) Tonic, gonorrhea, treatment diuretic

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Cleistopholis patens (Benth.) Engl. (Annonaceae)	Ojo	B B B B	Bark & root used for treatment of oedema (Ibuazizi), loss of appetite, malaria fever. Also used to prepare pepper soup for women after child birth	Infusion of bark used for infective hepatitis
Cnestus ferruginea DC (Connaraceae)	Atunkita; Mbe izi; utu nkita; Njunahu	R R R R	Root used to cure skin rashes. Fruits used to clean teeth. Root & leaves are remedy for filaria worm	Fruits, leaves roots: wounds, sore throat and gums
Combretum racemosum P. Beauv. (Combretaceae)	Mbaji, Ubi utu-ugo	R R	Root used for treating intestinal worms and other stomach complaints (Dysentery, etc.)	(Leaves, roots) Anthelmintic coughs remedy
Cucumis melo L. (Cucurbitaceae)	Bara (= Yoruba)	Fr	Used for treatment of gonorrhea (with other materials)	
Curcurma domestica Val. (Zingiberaceae)	Udo, odo	Rh Rh Rh Rh	Powered rhizome used as base materials	Malaria; used in skin diseases, also for sore eyes
Cyclicodiscus gabunensis (Taubert) Harms (Leguminosae)	Uzi	B B B B	Bark boiled & drunk by women after child delivery. Also used as a remedy for gonorrhea, cleaning stomach and body weakness	(Bark) Inhalant, enema for stomach pains, lotion for rheumatism

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Dennetia tripetala Bak. f. (Annonaceae)	Mmimi	Fr	Spice; stimulant	
Detarium senegalense J. Gmelin (Leguminosae)	Ofe, ofor	St St St St	Wood is used in invocation of spirits	Bronchial cough; febrifuge
Dialium guineense Willd. (Leguminosae)	Iceku, , Icheku, Aba cheche Aba-chiku	R	Unknown	Root bark used for stomach complaints of women
Dichapetalum sp. (Dichapetalaceae)	Akwu-ocha Akwu-osa	R	Decoction of root or other part of the plant poisonous to domestic and wild animals. Plant used as rat poison	
Enantia chlorantha Oliv. (Annonaceae)	Erume (=bark) Ike-agwu =root)	R R R R	Root strengthens the heart if chewed, infusion of root or bark cures malaria fever	(Bark) Topical on ulcers, antipyretic, haemostatic
Entada pursaetha DC (Leguminosae)		Sd Sd Sd Sd	Medicinal use of seed not revealed	Powdered seed used for fever
Erythrophleum ivorense A. Chev. & E. *E. suavolense* (Guillemin & Perrottet) Brenan (Leguminosae)	Inyi	B		Rodenticide healing rituals poisoning
Fagara macrophylla Engl. (Now = *Xanthoxylum macrophylla)* (Rutaceae)	Uko	R R R R	Treatment of gonorrhea, cures tuberculosis	Remedy for tooth ache, chewing stick

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part				Local Medicinal Uses	Known Medicinal Uses from Literature Sources
		U	OW	OL	E		
Garcinia kola Heckel (Guttiferae)	Aki-ilu	B		B		Leaves are used in treatment of sour breast milk in nursing mothers. Seed edible and used as a remedy for coughs. Bark used for malaria fever	Fruits used for inflammation of respiratory tract
Khaya spp. (Meliaceae)	Ulo, Egbuebe	B	B	B	B	For treatment of malaria fever, gonorrhea	Malaria, vermifuge
Kigelia africana (Lam.) Benth. (Bignoniaceae)	Oke-ogirisi			Fr	Fr	Fresh fruit infusion used treatment of gonorrhea, women problems	Externally for inflammation, venereal diseases
Maesobotrya barteri (Baill.) Hutch. (Euphorbiaceae)	Uvunne	R				Use of root not revealed	Bark used for diarrhea, as an abortificient, chewed as a stomachic
Milletia aboensis (Hook. f.) Bak. (Leguminosae)	Eze-chi Kpukpummaya	R				Used as laxative and treatment of constipation in children	
Milicia excelsa (Syn. *Chlorophora excelsa)* (Moraceae)	Oji	R		R		Tonic	Tonic inflammation, in nausea, exhaustion, elephantiasis, antiseptic (wounds)
Monodara myristica (Gaertn.) Dunal (Annonaceae)	Ehuru	Sd	Sd	Sd	Sd	Seed used as spice in pepper soup	Seed: condiment, stomachic Ext. Use: Guinea worm

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part				Local Medicinal Uses	Known Medicinal Uses from Literature Sources
		U	OW	OL	E		
Morinda lucida Benth. (Rubiaceae)	Mbobo, Isimkpi, Akamu, manu-ukwu	R		R		Root & leaves used for treatment of malaria fever	(Bark, root, & leaves) Emetic diuretic, purge astringent, malaria, yellow fever
Nauclea latifolia Merr. (Rubiaceae)	Uburu, Uburu-ilu Uburu-agu	R	R	R	R	Root & stem used to cure gonorrhea and women problems such as infertility. All parts of plant used for treatment of malaria fever	Hemorrhoids purge, tonic, antimalarial, stomachic, diuretic, coughs
Newbouldia laevis Seeman ex Breau (Bignoniaceae)	Ogirishi Umune	R				Bark boiled with other herbs, stops vomiting	Bark used as anthelmintic stomachic & anti-convulsion, febrifuge
Pentaclethra macrophylla Benth. (Leguminosae)	Ugba, Ugbake	B		B		Bark used for treatment of skin diseases, water extract of bark taken by women after child birth	Stem bark is astringent anthelmintic and used topically in treatment of psoriasis
Petersianthus africanus Merr. (Syn. *Combretodendron africanum)* (Lecythidaceae)	Anwusi Anyasi			B			Expectorant (barks)
Picralima-nitida (Stapf.) Th. & Dur. (Apocynaceae)	Osu Nkpokiri	Fr	Fr	Fr	Fr	Fruit, seed root & other parts are used in treatment of malaria fever, gonorrhea and women problems	

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Piper guinensis Schum. & Thonn. (Piperaceae)	Uziza	Fr Fr Fr Fr	Spice. Soup made with piper fruit Xylopia (Uda). Fruit is taken by women after child birth. Fruit used as one of ingredients in preparation for curing hepatitis (jaundice-Iba oji)	Aromatic rheumatism rubefacient
Piptadeniastrum africanum Brenan (Leguminosae)	Ughi Uchi	B	Medicinal uses of stem barks not revealed	Bark used for headache, for toothache, as abortifacient
Pterocarpus soyauxi Taub. (Leguminosae)	Uchie-awala; Awala	W W W W	Cream from heart wood (Uchie) used as base material in preparation of native medicine for skin diseases	
Rauwolfia vomitoria Afz (Apocynaceae)	Akanta, Akata, Ogwu-oyi	R R R R	For gonorrhea	Sedative, emetic
Ricinodendron heudelotii (Baillon) Pax (Euphorbiaceae)	Okwe	Sd Sd Sd Sd		(Leaves, root seed, latex-) Antiabortive, febrifuge
Securidaea longipedunculata Fres. (Polygalaceae)	Uga-agu	R R R R	Root for treating coughs, gonorrhea, tuberculosis, diarrhea, convulsion in children	Root & bark: drastic purge, expectorant, diaphoretic, diuretic, taeniacide

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Strophanthus hispidus DC (Apocynaceae)	Osisi-Kaguru or Osisi-Nkeaguru	R R R R	Root for treatment of gonorrhea, women complaints (infertility), waist pains, malaria fever, skin diseases (rashes, itch) and many other ailments, (rheumatism, etc.)	Roots: venereal diseases, rheumatism, intestinal parasites, as diuretic and for urethral discharge, syphilis
Tetrapleura tetraptera Benth.		Fr Fr Fr Fr	Fruit used as one of the materials for convulsion medicine, and stomach complaints. Medicinal use of root not revealed	Fruit used for rheumatism
Uvaria chamae P. Beauv. (Annonaceae)	Mmimi ohia,	R R R R	Fruit edible, root for treating gonorrhea, tuberculosis, malaria fever, women problems, skin diseases, crushed root used as counter irritant	Root bark used internally for cataarrhal inflammation of mucous membranes, Yellow fever, stomachic, vermifuge
Xylopia aethiopica (Dunal) A. Rich (Annonaceae)	Uda	Fr Fr Fr Fr	Fruit is one of the materials in herbal medicine preparations for hepatitis, women complaints (infertility), convulsion and stomach problems	Fruit used for coughs as carminative purgative

Appendix II (Continued)

Scientific Name	Vernacular (Igbo) Name	Place (Market) & Plant Part U OW OL E	Local Medicinal Uses	Known Medicinal Uses from Literature Sources
Zanthoxylum macrophylla Oliver (Syn. *Fagara macrophylla* (Rutaceae)	Uko	R R R R	Remedy for gonorrhea, tuberculosis	Urinary antiseptic, gonorrhea (VD), coughs, rubefacient, toothache, chewing stick
Zingiber officinale Rosc. (Zingiberaceae)		R R R R	Rhizome (half dry) chewed as stimulant	Stimulant, aromatic, taken for indigestion, fever etc.

Key to abbreviations of plants parts: B = Bark; Fr = Fruit; Lf = Leaf; R = Root; Rh = Rhizome; Sd = Seed; St = Stem; Wd = Wood

Appendix III. Commonly Exploited Plant Species in the Northeastern States. Source: Data from ethnobotanical survey of Northeastern States, Nov. 1991.

Botanical Names Family	Vernacular Names (Hausa)	Parts used	Medicinal Uses
Acacia nilotica (L.) ex Del. (Leguminosae)	Gabaaruwaa	Fruit	Swollen parts of body, leprosy
Anogeissus leiocarpus Guill. & Perr. (Combretaceae)	Markee	Stem bark	Worm expeller
Aristolochia albida Dichartre (Aristolochiaceae)	Duma duwtsee	Root	For syphilis
Bosweillia dalzielli Roxb. ex Colebr. (Burseraceae)	Ararrabii		For bodily swelling, jaundice
Cissus quadrangula L. (Vitaceae)	Gwaye tsaniya	Leaves	Urinary tract infection
Detarium microcarpum Guill. Perr. (Leguminosae)	Taura	Stem bark	Anti-hypertensive
Erythrinia senegalensis DC. (Leguminosae)	Majiriyaa	Stem bark	Jaundice
Erythophleum guineense G. Don (Leguminosae)	Gwaska	Stem bark	Headache

Appendix III. (Continued)

Botanical Names Family	Vernacular Names (Hausa)	Parts used	Medicinal Uses
Guiera senegalensis J.F. Gmel. (Combretaceae)	Saababaraa	Leaves	For dysentery
Isoberlina doka Craib. Stapf (Leguminosae)	Dookar	Stem bark	Snake bite
Mitracarpus scaber Zucc. (Rubiaceae)	Googa maasu	Leaves	For swollen spleen
Mitragyna inermis (Willd.) O. Kitze (Rubiaceae)	Gryayaa	Stem bark	Arthritis
Mormordica charantia L. (Curcurbitaceae)	Garahuni	Leaves	For measles
Pterocarpus erinaceus Poir (Leguminosae)	Madobiyaa	Stem bark	For inducing breast milk
Schweinkia americana L. (Solanceae)	Danana	Root	Constipation
Sterculia setigera Del. (Sterculiaceae)	Kukkuki	Stem bark	Anemia
Striga spinosa (Del.) Benth. (Scrophulariaceae)	Makasha	Leaves	For stroke
Strychnos spinosa Lam. (Logniaceae)	Kookuyaa	Root bark	For jaundice
Tephrosia purpurea (L.) Pers. (Leguminosae)	Magaguinar	Root	For treatment of stomach and breast problems
Terminalia glaucescens Planch. ex Benth. (Combretaceae)	Bashee	Stem bark	Backache
Vitellaria paradoxa Gaertn. f. *Butyropermum paradoxum* (Sapotaceae)	Kandaya	Stem bark	Rabies
Waltheria indica L. (Sterculiaceae)	Yankufa	Whole plant	Anemia, stomach swelling in children
Zingiber officinale Rosc. (Zingiberaceae)	Cittarfo	Rhizome	Aphrodisiac

Appendix IV. Medicinal Plants in Cultivation in Medicinal Plant Gardens in Southeastern Nigeria. Source: Gbile & Onyeachusim (1990).

Scientific Name	Some known recorded medicinal uses from literature and field research
Abrus precatorius L. (Leguminosae)	Plant used for malaria, kidney and bladder troubles: root chewed as aphrodisiac, used to cure sore throat and rheumatism, etc.
Acanthus montanus (Nees) T. Anders (Acanthaceae)	Root bark pounded and pasted over boils and abcesses to bring them to a head and make them burst in consequence (local use in Orlu area)
Aframomum melagueta Schumann (Zingiberaceae)	Seed used as spice, vermifuge, stimulating carminative, domestic remedy and in veterinary medicine
Bryophyllum pinnatum (Lam.) Oken (Crassulaceae)	The root is used in cough medicine. The juice is used as a diuretic and applied for ear ache and opthalmia; leaf juice (extracted by softening the leaf in hot water) is used locally for navel cord treatment in newly born children
Cissampelos owariensis P. Beauv. (Menispermaceae)	Roots used in fever and diarrhea. Leaves used in local application for itching and leaf decoction as a light purge. Root used as emenagogue. (Also see Appendix I)
Cissus quadrangularis Linn. (Vitaceae)	Uses not disclosed
Cryptolepis sanguinolenta (Lindl.) Schtlr. (Periplocaceae)	Leaves used for treating fatigue. Root infusion used in treatment of stomach and intestinal disorders and as an anti-rheumatic agent. Aqueous extract of roots said to be effective in treating malaria and urinogenital infections
Hymendictyon pachyantha K. Krause (Rubiaceae)	Boiled leaf, root and stem drunk to kill and expel round worms; leaf decoction drunk to cure convulsions and used as mouthwash
Mondia whitel (Hook f.) Skeels (Periplocaceae)	Uses not disclosed
Ocimum gratissimum Linn. (Labiatae)	Leaf infusion is a remedy for fever of all kinds. Leaf decoction is regarded as stomachic and laxative and is used for coughs and conjuctivitis
Pyrenancantha staudtii Engl. (Icacinaceae)	Leaf decoction is used for treating diabetes

Appendix IV (Continued)

Scientific Name	Some known recorded medicinal uses from literature and field research
Rhaphiostylis beniniensis Planch ex Benth. (Icacinaceae)	Decoction of leaf, root and stem is drunk to kill and expel round worm; leaf decoction drunk to cure convulsion and is used as mouthwash and lotion for sores. Root and stem infusion used as tonic for children (2-3 yrs.). Infusion of root and stem is used by pregnant women to strengthen their offspring
Senecio biafrae Oliv. & Hiern (Compositae or Asteraceae)	Pepper soup prepared with the leaves is drunk to cure coughs (Local medicinal uses at Orlu, Imo State)
Sphenocentrum jollyanum Pierre (Menispermaceae)	Root chewed to cure loss of appetite, stomach pains constipation; leaf & bark used for wound dressing (healing); fruit stewed with lime juice used to cure coughs

PRODUCTION OF ABNORMAL SOMATIC EMBRYOS AND THEIR TRANSFERRING INTO NORMAL SEEDLINGS IN COTTON TISSUE CULTURE

Zheng Sijun
Department of Agronomy, Zhejiang Agricultural University
Hangzhou, 310029 P. R. China

SUMMARY

Embryogenic callus was initiated and suspension cell cultures were established with hypocotyls of *Gossypium hirsutum* L. cv. Coker 312 and Coker 201. The frequency of somatic embryo induction was the highest in medium MS + B_5 + 0.1 mgl^{-1} ZT. Along with production of normal somatic embryos, many abnormal embryos were also produced in somatic embryogenesis. There were six types of abnormal embryos. Abnormal embryos could be transformed into normal seedlings when they were placed in germination medium SH + 0.5 mgl^{-1} IAA + 0.5 mgl^{-1} BAP + 0.025% active + 0.2% Gelrite. Two or more buds could be produced from some single embryos. The mean conversion from embryo germination into plant regeneration was 35.25%. Organogenesis was also observed in cotton tissue culture.

INTRODUCTION

Tissue culture of cotton is more difficult than that of other crops. It was not until in 1983 that Davidonis and Hamilton (1983) reported plant regeneration from cotyledon derived callus subcultured for two years. Since then, many authors (Chen and Li , 1987; Finer, 1988; Li and Wen, 1989; Li et al., 1991; Rangan, 1984; Trolinder and Goodin, 1988a,b; Trolinder and Chen, 1989; Zhang and Wang, 1989) reported somatic embryogenesis of *Gossypium hirsutum* L. Guo and Jiao (1988) and Zheng (1991) observed that chromosomal variations were easily produced in embryogenic cell subcultures. Many abnormal embryos were induced in somatic embryogenesis. The conversion rate from embryo germination into plant regeneration was very low. This phenomenon influenced further utilization of the technique for cotton improvement. In this study, the types of abnormal embryos and their cause in somatic embryogenesis were analyzed. The method for transforming abnormal embryos into normal seedlings was also suggested.

MATERIALS AND METHODS

Seeds of *G. hirsutum* L. cv. Coker 312 and Coker 201 were delinted with sulfuric acid and then surface-sterilized for 30 min in 0.1% $HgCl_2$. The seeds were rinsed 3 times in sterile water and placed on a medium containing 1/2 MS salts, 1% sucrose, 0.8% agar, pH 6.5. Seedlings were obtained in 3 days. 3-5 mm sterile hypocotyl sections of two cultivars were used as explants. The basal medium for callus induction was MS salts with B_5 vitamins, supplemented with 2.0 mgl^{-1} IAA and 0.1 mgl^{-1} KT, glucose 3%, agar 0.8%, pH 5.8. The medium for the callus subculture was the same as the callus induction medium. Suspension culture medium was MS + B_5 + 1900 mgl^{-1} KNO_3 + 15 mM glutamine. The medium for somatic embryo induction was MS salts with B_5 vitamins, supplemented with different growth regulator regimes. Two kinds of media were used for somatic embryo germination. Medium I was the same as

callus induction medium, but without hormones. Medium II was SH medium (Steward and Hsu, 1977), supplemented with 0.5 mgl^{-1} IAA, 0.5 mgl^{-1} BAP, 0.025% active carbon, 0.2% Gelrite, pH 6.5.

The morphological change from callus to the somatic embryo process was investigated by Zoom microscopy. For histological studies, embryogenic callus and somatic embryos at different developmental stages were fixed in FAA fluid, then dehydrated in a graded series of alcohol, cleaned in xylene and embedded in paraffin wax. Sections of 10 μm thick were cut and stained with 1% Safaranin and 0.5% Fast Green. Observation and photographs were made on Olympus BH-2 research microscope.

RESULTS AND DISCUSSION

1. Induction of somatic embryos

In suspension cultures of Coker 312 , embryogenic callus could be quickly proliferated and somatic embryos at different developmental stages were produced after 2-3 weeks. When these somatic embryos, obtained from liquid suspension culture medium, were transferred to solid medium, they were readily evolved to callus-like and seldom germinated into plants. After suspensions were sieved, those suspensions which were larger than 60 mesh were discarded. Suspensions of smaller cell clusters, homogenous in size were collected and transferred to MS with B_5 vitamins medium, supplemented with different regimes of growth regulators and active carbon for somatic embryo induction (Figure 1).

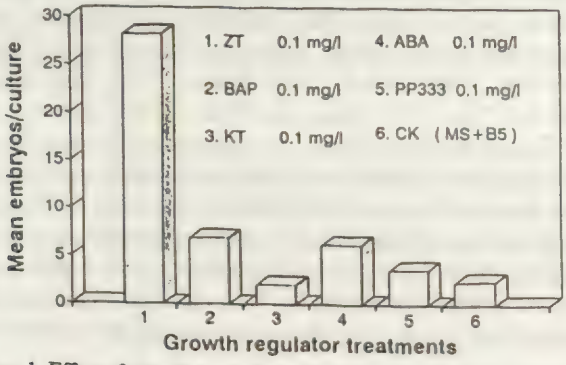


Figure 1. Effect of growth regulator regimes on somatic embryo induction.

Results showed there were significant differences in somatic embryos induction frequency in different growth regulator regimes. Cytokinin ZT was much better than abscisic acid and paclobutrazol. ZT was the most effective cytokinin. The mean somatic embryo number per culture was 28.1. Somatic embryos were recorded as torpedo shape or cotyledonary stages after one month. As for cytokinin BAP, the mean somatic embryo number per culture was 6.9, as was the ABA treatment. Cytokinin KT was similar to CK. PP333 showed small effects for somatic embryo induction. These results indicate that ZT had a specific effect on somatic embryo induction from embryogenic suspension cell cultures. Many somatic embryos with globular shape, heart shape, torpedo shape and cotyledon stage were formed from embryogenic cell masses (Plate I, 1-5).

Plate I. 1-18. Somatic embryogenesis and plant regeneration, abnormal somatic embryos and abnormal seedlings in *G. hirsutum* L. 1. Embryogenic callus (x 5). Globular shaped embryo (x 15). 3. Heart shaped embryo (x 15). 4. Torpedo shaped embryo (x 15). 5. Cotyledon embryo (x 12.5). 6. Regenerated plant (x 0.43). 7. Embryo evolved to callus-like (x 3.5). 8. Vitreous embryo(x 5). 9-11. Longitudinal section of combined embryos (x 30, x 30, x 75). 12. Multi-combined embryos (x 5). 13-14. Embryo of abnormal cotyledon with single and multi-cotyledon, respectively (x 5, x 3.75). 15. Embryo without bud and root (x 10). 16-17. Abnormal seedlings (x 10, x 0.4). 18. Regenerated plants with four buds from an embryo (x 0.46).

2. Types of abnormal somatic embryos and their transferring into normal seedlings

In addition to normal somatic embryos, many abnormal somatic embryos were produced in embryogenesis of the two cultivars (Plate I, 7-15). There were six types of abnormal somatic embryos; their characters are listed in Table 1.

Combined embryos could be further divided into two kinds, primary combined embryos (Plate I, 9-10,12) and secondary combined embryos (Plate I, 11). Combined embryos were multiple embryos that were attached at the suspensor or cortex of hypocotyls. Combined embryos with a little attachment could be separated by shaking or cleaving, but, it was not easy to separate those combined embryos with a broad-based

Table 1. Characteristics of different types of abnormal somatic embryos.

Types	Characters of abnormal somatic embryos
1. Embryo evolved to callus like	Callus was again induced from somatic embryos
2. Vitreous embryo	Embryos were vitreous and hard
3. Combined embryo	Two or more than two embryos were combined
4. Embryo of abnormal cotyledon	Embryos without cotyledon or with single or with multi-cotyledon
5. Embryo of embryonal axis	Embryos without bud, root and cotyledon only embryonal axis
6. Embryos with polybud	Embryos with three or more than three buds

attachment. Secondary combined embryos were produced from original embryos. In that case, a larger embryo was attached along with a smaller embryo.

When somatic embryos developed into the cotyledon stage, fully developed embryos were germinated and the resulting seedlings grew into plants when transferred to the germination medium (Plate I, 6). In general, buds and roots of developed embryos enlarged and grew in the same time. Regenerated plants were obtained, but there were many abnormal seedlings obtained from somatic embryos. Sometimes a bud was formed without a root (Plate I, 16). In other cases, main roots grew first and secondary roots were also produced, but seedlings grew slowly (Plate I, 17). Eventually, they stopped growth. That is the principal reason that there were very low conversions from somatic embryo germination into plant regeneration.

Some abnormal somatic embryos, such as combined embryo, embryo with polybud and vitreous embryo, could germinate and grow into plants when they were transferred into germination medium II. For Coker 312 and Coker 201, the conversion percentage was 38.04 and 32.47, respectively. Whereas in germination medium I, the conversion percentage was only 19.11 and 11.51, respectively (Figure 2). Medium II was much better than medium I for somatic embryos germination. In medium II, 2-4 buds could be produced from some single embryos. It appears that organogenesis may occur in cotton tissue culture. At least, somatic embryogenesis was incompletely developed.

3. Production of abnormal somatic embryos and abnormal seedlings

Chromosomal variations are expected to create genetic variability. This is very important for crop improvement. Cytological observation of Coker 312 suspensions subcultured more than 6 months indicated that variations of chromosomal number and chromosomal structure were produced in embryogenic cells. A wide range of chromosome numbers were observed, varying from diploid to hexaploid in euploid and from 16 to more than 78 in aneuploid. There were several mitotic abnormalities, such as multiple nuclei and micronuclei in interphase, chromosome ring and telophase bridge in

mitotic stage (Zheng, 1991). Chromosomal variation in embryogenic callus cells was one of the main reasons which caused abnormal somatic embryos and abnormal seedlings. To overcome this problem, it is better to use short subculturing suspensions for somatic embryogenesis. In addition, it was necessary to prohibit somatic embryos from premature germination (the other reason for production of abnormal somatic embryo). When these methods mentioned above were used, we obtained many normal

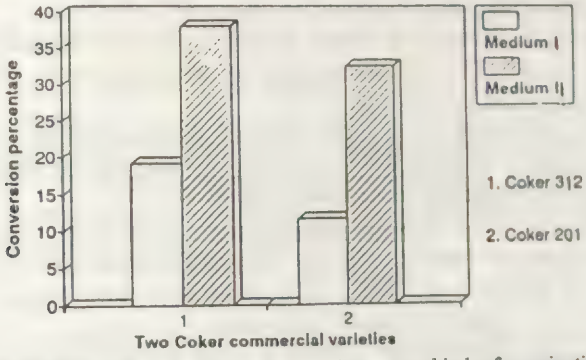


Figure 2. Comparison of somatic embryo germination on two kinds of germination media for two Coker varieties.

somatic embryos. However, it was necessary to use appropriate regimes and concentrations of auxins and cytokinins for transforming abnormal embryos into normal seedlings.

It was very easy for callus initiation in cotton tissue culture, but until now, somatic embryogenesis and plant regeneration were obtained in only a few genotypes. The conversion from embryo germination into plant regeneration has been very low, this being due to the production of large numbers of abnormal somatic embryos in embryogenesis. We recommend that for induction conditions in embryogenesis, the methods of plant regeneration and techniques for somatic embryo germination need additional study.

ACKNOWLEDGEMENTS

This research was supported by funds from International Foundation for Science (C/1654-1). Thanks to Professors Ji Daofan and Xu Fuhua for kind supervision.

REFERENCES

Chen Zhixian and Li Shujun. 1987. Some characteristics of somatic embryogenesis and plant regeneration in cotton cell suspension culture. Scientia Agricultura Sinica 20:6-11.

Davidonis, G.H. and Hamilton, R.H. 1983. Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Science Letter 32:89-93.

Finer, J.J. 1988. Plant regeneration from somatic embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.). Plant Cell Reports 7:399-402.

Guo Xiangmei and Jiao Gaili. 1988. Chromosomal variation in cell culture of upland cotton (*Gossypium hirsutum*). China Cottons 15:12-13.

Li Xiulan and Wen Lanying. 1989. Cotton tissue culture and plant regeneration. China Cottons 16:13-15.

Li Xiulan, Li Fenglian and Li Fuguang. 1991. Transfer of abnormal seedlings to normal ones in cotton (*Gossypium hirsutum* L.) tissue culture. Acta Agronomica Sinica 17:392-393.

Rangan, T.S. 1984. Somatic embryogenesis in tissue culture of *Gossypium hirsutum* L. In Vitro 20:156.

Steward, J.M. and Hsu, C.L. 1977. In-ovulo embryo culture and seedling development of cotton (*Gossypium hirsutum* L.). Planta 137:113-117.

Trolinder, N.L. and Goodin, J.R. 1988a. Somatic embryogenesis in cotton (*Gossypium hirsutum*) I. Effect of source explant and hormone regime. Plant Cell, Tissue and Organ Culture 12:31-41.

Trolinder, N.L. and Goodin, J.R. 1988b. Somatic embryogenesis in cotton (*Gossypium hirsutum)* II. Requirements for embryo development and plant regeneration. Plant Cell, Tissue and Organ Culture 12:43-53.

Trolinder, N.L. and Chen Zhixian. 1989. Genotype specificity of the somatic embryogenesis response in cotton. Plant Cell Reports 8:133-136.

Zhang Dali and Wang Zhezhi. 1989. Tissue culture and embryogenesis of *Gossypium hirsutum* L. Acta Bot. Sinica 31:161-163.

Zheng Sijun. 1991. Chromosome variation in callus culture of *Gossypium hirsutum* L., *in:* "Conservation of Plant Genes, DNA Banking and *in vitro* Biotechnology," R.P. Adams and J.E. Adams, eds., Academic Press, San Diego, CA.

DECISION POINTS FOR TRANSFERRING PLANT INTELLECTUAL PROPERTY TO THE PRIVATE SECTOR

Steven C. Price and Leanna M. Lamola
Iowa State University Research Foundation, 214 Office and Lab
Iowa State University, Ames, Iowa 50010, U.S.A.

SUMMARY

The flow of intellectual property is discussed in terms of contracts, plant patents, the plant variety protection act, utility patents, seed certification, foundation seed organizations, branding and "variety not stated", and licensing. Licensing agreements can include the ability to conduct research, access to germplasm, geographical limitations, local plant restrictions and royalty distributions. General decision trees are presented as models for the transfer of intellectual property.

INTRODUCTION

The purpose of this article is to discuss two complementary technology transfer systems for plant intellectual property, and to discuss various decision points to be considered along the path from discovery to royalty generation. The history of the seed industry/plant breeding/variety release in any country begins with public sector initiatives concerned with quality control. In the US, the first government investments in plant breeding followed the establishment of the land grant colleges and state agriculture experiment stations through the 1862 Morrill Act, and the 1887 Hatch Act. Since that time major efforts have been undertaken at US institutions to introduce and improve crop varieties for US agriculture. Because public money supported this work, new varieties were released free of charge to farmers and farmer organizations.

However, many universities in the United States are retreating from traditional policies that release new plant varieties to the public free of charge. Universities find themselves in this new posture because of deteriorating local economic situations, the need to supplement research budgets, and a growing awareness of the need to handle plant varieties in keeping with practices which will maximize public usage and economic return. Obtaining intellectual property rights to new varieties and subsequently licensing them for royalties has become increasingly more common. This also reflects the sentiment of several technology transfer laws that were passed in the 1980s which give the right of inventors and institutions to share in royalties generated from federal funds.

These new activities have met with considerable resistance from parties that have been accustomed to releasing plant varieties free and unrestrained. Indeed, many believe that proprietary concerns will interfere with scientific exchanges of genetic material with a resulting decrease in the advancement of science.

Concerns about access to genetic resources have been recently brought to the public's attention as a "hot button" issue by the June, 1992 United Nations Earth Summit in Rio de Janeiro, Brazil. 150 countries, excluding the United States, signed the *Convention on Biological Diversity*, whose objectives were:

". . .conservation of biological diversity. . .the sustainable use of its components. and the fair and equitable sharing of the benefits arising out of the utilization of genetic resources. . .including. . .appropriate access to genetic resources. . .appropriate transfer of relevant technologies. . .and by appropriate funding."

Article 15 (7) states that:

"Each contracting party shall take legislative, administrative. . .measures. . .with the aim of sharing in a fair and equitable way the results of research and development and the benefits arising from the commercial and other utilization of genetic resources . . .upon mutually agreed terms."

The language of the Convention clearly acknowledges the desire for sharing monetary benefits, and recognizes the need for intellectual property protection as being important for this end. Article 16(2) states:

"Access to and transfer of technology. . .shall be provided and/or facilitated under fair and most favorable terms, including on concessional and preferential terms where mutually agreed. . .

In the case of patents and other intellectual property rights, such access. . .shall be provided on terms which recognize. . .adequate. . .protection of intellectual property rights."

Various passages in the Convention raised the specter of "compulsory licensing" and uncertain royalty obligations; these uncertainties led many biotechnology companies to oppose the signing of the Convention, and certainly influenced the Bush Administration's decision not to do so. However, the movement of developing nations towards a more business-like approach for the disposition of their genetic resources will require application of the same intellectual property right techniques and instruments that industry itself has used for hundreds of years.

It is evident that the world is moving towards a system that is more dependent on contracts, licensing, and intellectual property protection. A vision of the future can be seen by an agreement reached by the Instituto Nacional de Biodiversidad (INBio) in Costa Rica, a private, non-profit organization, chartered in 1989 for "purpose of putting biodiversity to work for Costa Rican society." INBio signed an agreement with Merck for a two year, non- exclusive, license, for US $1,135,000. Under the agreement, INBio supplies a limited number of biological samples for genetic screening, INBio participates in royalties from Merck on products originating from the samples, and royalties received by INBio will be split 50/50 with the Costa Rican National Park System. Other developing countries are looking at the INBio-Merck arrangement as model.

Managers of plant intellectual property in both developed and developing countries are finding it necessary to learn about modes of intellectual property protection that are foreign to their past practices. For example, in the United States the application of many instruments such as confidentiality agreements, material transfer agreements, utility patents, and licenses, which are normal, routine, and customary from fields outside of plant breeding, may be alien to managers of plant intellectual property. Conversely, managers of technologies outside of plant breeding are equally unfamiliar with traditional systems employing seed certification, foundation seed organizations, plant patents, plant variety certificates, and varietal naming.

The movement of many novel plant biotechnologies through university and other nonprofit channels will require the confluence of these two technology transfer systems. The melding of patents, licensing, confidentiality agreements, material transfer agreements, with the traditional structure of Plant Variety Protection, seed certification, and foundation seed organizations will be increasingly more common. From Figure 1, we distinguish three non-mutually exclusive decision points:

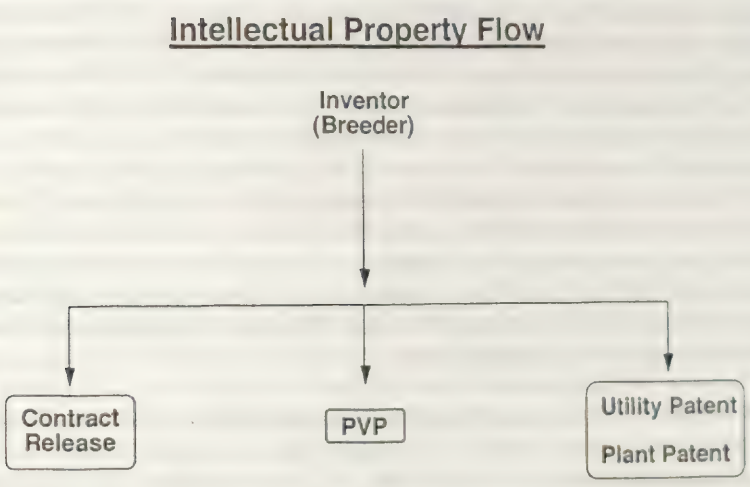


Figure 1. Diagram of the decision flow for plant intellectual property.

PLANT INTELLECTUAL PROPERTY: CONTRACTS

Plant varieties are protectable as "trade secrets," and can be released on a contract basis to users using terms that are similar to license agreements (below). Such releases have the advantages of timeliness and low cost. However, disadvantages result from the strength of the protection being only as good as the laws in the state or country in which the terms of the contract are governed.

PLANT INTELLECTUAL PROPERTY: PLANT PATENTS

Before the 1920s, there was little private investment in applied breeding/variety development in the US because it was impossible for the private breeder to maintain control over a new variety once it was marketed. This is because vegetatively-propagated and open-pollinated varieties can be easily multiplied, renamed and sold. Without exclusive control, the private firm was not able to recoup the costs of variety development.

Interestingly, private cereal and fruit breeders lobbied for a plant patent system as early as 1885. In 1905, plant patent legislation was considered but never progressed in a House Committee.

The development of hybrid maize in the 1920s provided an effective means to maintain exclusive control over seed production and marketing. Hybrid seed is a trade secret, because it is the result of the controlled cross of two inbred parents, the identity of which may be held secret. As public universities began to develop and release inbreds free to the public, many start-up seed companies were established to create and market hybrid seed developed from inbreds. Eventually, successful private firms began to develop their own inbreds. However, the extent of private investment in non-hybrid variety development remained limited because of the aforementioned limitations on exclusivity.

The first form of statutory intellectual property protection for plants was a 1930 amendment to the utility patent statute called the Plant Patent Act (PPA) (35 USC

section 161). Under the PPA, a breeder who develops an asexually-reproduced variety (with the exception of uncultivated and tuberous species) that is novel, distinct and unobvious will receive patent protection. Action taken by the USDA, however, prohibited inclusion of sexually-reproduced varieties in the act. The USDA successfully argued that because sexually-reproduced species were genetically unstable, a patented variety would eventually differ from its patent disclosure, rendering the patent unenforceable.

One of the most active lobbyists for passage of this act was Thomas Edison, who acted on behalf of his deceased friend, Luther Burbank, the prolific plant breeder and inventor of hundreds of new fruit and vegetable varieties. After the act was signed into law, Edison told the New York Times, "Burbank would have been a rich man today if he had been protected by such a patent bill." Since 1930, over 6000 plant patents have been issued by the Patent and Trademark Office.

PLANT INTELLECTUAL PROPERTY: PLANT VARIETY PROTECTION ACT

Intellectual property protection of sexually-reproduced plant species was not seriously considered in the US until the 1950s. By that time, private industry interest in breeding self-pollinated crops had substantially increased. A number of private firms had initiated successful marketing efforts based on *uncertified, branded seed*. Additionally, several European countries had implemented successful plant breeders' rights laws for sexually-reproduced species. Moreover, in 1961, six European nations formed the Union for the Protection of New Varieties of Plants (UPOV) to provide an international framework for national plant breeder rights legislation; this was called the UPOV Convention.

In 1969, the American Seed Trade Association introduced a bill to Congress based on European plant breeders' rights laws. The bill was successfully passed into law the following year as the Plant Variety Protection Act (PVPA). Under the PVPA, the USDA will issue an eighteen-year Plant Variety Protection Certificate to the developer of a sexually-reproduced variety that exhibits novelty, uniformity and stability.

Enactment of the PVPA also added Title V to the Federal Seed Act. Title V states that it is against the law to sell seed ". . .by variety name, seed not certified by an official seed certifying agency, when it is a variety for which a certificate of plant variety protection under the Plant Variety Protection Act specifies sale only as a class of certified seed. . ." This very awkward clause has unfortunately been misinterpreted. The clause means that if Title V is selected by the originator, certified seed must bear the name of the variety stated in the Plant Variety Protection Certificate. However, it does not mean that the election of Title V prohibits selling the seed under another name (and therefore branded) if it is non-certified.

Although the PVPA was modeled on the utility patent statute, it provides less protection because of two exemptions. The "research exemption" allows breeders to use protected varieties for research purposes and to develop new varieties. The "farmers' exemption" allows a farmer who is primarily a commodity producer to plant the progeny seed of a purchased protected variety on the farm or sell it to other farmers.

PLANT INTELLECTUAL PROPERTY: UTILITY PATENTS

It is generally recognized that the strongest form of intellectual property protection that can be offered is that of a utility patent. A utility patent is essentially a legal right that permits the owner to exclude others from making, using, and selling an

invention. The exclusionary right lasts for 17 years in the U.S., and for a similar period in other parts of the world, after the patent is issued. A patentable invention has four essential elements: it must cover certain *subject matter*; it must be *useful*; it must be *novel;* and it must be an *unobvious extension* of what is already known to the public. A patent has two notable features: it enables an inventor to teach the world (it is not a secret); and it gives a monopoly on its use to the inventor (more accurately, the title holder). In return for this monopoly, the inventor must disclose his or her invention to the world in such detail that other people will be able to make and use it (enablement). For living biological materials such as seeds, such enablement is satisfied by making a deposit of the seed in an internationally recognized seed depository.

Utility patent protection for plants was established in the 1980s through two landmark court decisions. In *Diamond v. Charkrabarty*, the US Supreme Court held that genetically modified microorganism was patentable subject matter under the utility patent statute. In *Ex parte Hibberd,* the Patent and Trademark Office's Board of Appeals followed this precedent and granted a patent for a plant variety.

SEED CERTIFICATION

Before the turn of the century it was difficult to find supplies of pure seed of new varieties which had been produced, tested and released by the state experimental stations. Moreover, the general concept of varietal purity was not widely appreciated by farmers and seedsmen. Within three to four years of release a new variety would generally lose its genetic identity and become worthless to the farmer. Also, new introductions were often renamed by those who used and sold them. For example, the USDA reported that a variety of wheat called "Futz" that was distributed by USDA in 1871 was being grown a few years later under 24 different names. The main reason for this situation was that there were no standard procedures for insuring the production and distribution of new varieties under a single name and in genetically pure lots.

Around the turn of the century, former students of the land grant colleges began to form "crop improvement associations" to aid the agricultural experimental stations by coordinating seed increases of newly released varieties. Some crop improvement associations assisted those who increased seed by inspecting their fields before harvest. Canada pioneered this service as early as 1905. The first field inspections in the US were made around in 1913 in Wisconsin. Crop improvement associations soon began to develop their own seed inspection standards based on the results of inspections.

In 1919, representatives from crop improvement organizations in the US and Canada formed the International Crop Improvement Association (ICIA). One of the main objectives of this association was to provide a forum in which the seed improvement and certification methods of all member crop improvement organizations could be standardized. By 1921 the ICIA had begun to standardize methods of inspection and certification for a number of important crops. In 1929, it approved standard nomenclature for recognized seed classes - *foundation, registered and certified* (defined below and Figure 2).

In 1939, the Federal Seed Act was enacted to regulate the movement of certified seed through interstate commerce. The law recognized the two commercial classes of certified seed developed by the ICIA - registered and certified. Regulations promulgated under the act stated that only officially-recognized state agencies could certify seed. In Iowa, for example, the Iowa Crop Improvement Association is the only state certification agency. It is organized as a non-profit corporation and must perform certain

duties to retain recognition by the state secretary of agriculture. The Federal Seed Act did not provide a technical definition of "certified seed." It simply stated that certified seed was that which met a state certification agency's own rules. Nor was there was an ICIA rule that required member certification agencies to comply with ICIA standards. Therefore, differences remained between standards of state certification agencies.

The Federal Seed Act was amended in 1969 to deal with differing standards among state certification agencies. The amendments established genetic purity as the minimum basis for certification by an officially-recognized certification agency. Certification agencies could either certify all seed for genetic purity only or could also impose additional seed quality standards. The amendments also required all certified seed to bear a label issued by the official certification agency depicting the variety and class.

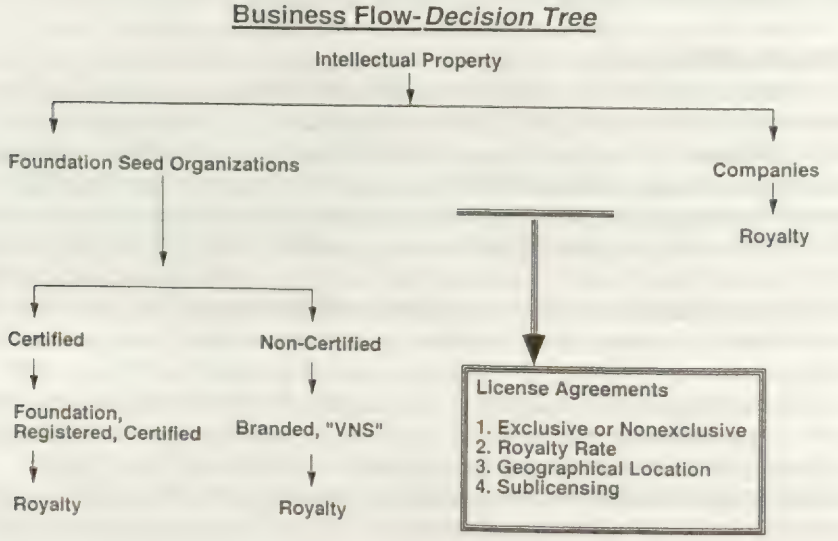


Figure 2. Diagram of business flow involving the intellectual property, whether protected by contracts, plant variety certificates, or patents.

Today, the classes of seed used by certification agencies are breeder, foundation, registered, and certified seed, defined as follows: **breeder** seed is from the developer or breeder; **foundation** seed originates from breeder seed; **registered** seed is grown from foundation seed and cleaned by the originator or an approved seed conditioner, and bagged, tagged, and sealed when sold; **certified** seed can be grown either from registered or foundation seed, cleaned by the originator or an approved seed conditioner, and bagged, tagged, and sealed when sold.

State seed laws may go beyond the Federal Seed Law and require that all seed, whether certified or not, be labeled under one variety name only. Such is the case in Minnesota. Today, most states today have an official seed certification agency, that

inspects fields for purity and regulates the production and labeling of foundation, registered, and certified seed.

However, with the growth of the private sector, many companies now have their own in-house quality control programs. The present trend could lead to a time when all varieties sold may be privately developed and noncertified. Attitudes of both large and small seed companies to certification vary. For some companies, there may be a continuing niche for seed certification, such as with varieties for specialty markets.

FOUNDATION SEED ORGANIZATIONS

Many foundation seed organizations in the US were organized in the 1930s and 1940s to maintain and increase the foundation classes of seed developed by the state agricultural experimental stations or the United States Department of Agriculture, and to provide a service for interregional testing. Public releases of new varieties from universities have been mainly through foundation seed organizations.

The nature of foundation seed associations varies widely from state to state. A foundation seed association may be a private, for-profit organization that operates independently of a state experimental station; it may be a non-profit organization connected to a state experimental station; or it may be an integral part of the state crop improvement association.

BRANDING AND "VARIETY NOT STATED"

Varietal naming can be rather complicated. A variety must be given a name that distinguishes it from other varieties of the same species. Normally, the originator gives the first name; a variety with a name is a "named variety." A basic decision is whether or not a variety will be allowed to be marketed using a company's name, i.e., "branded," as opposed to being marketed by the name given it by the originator. If a named variety is branded by a company, it must bear the label "variety not stated," (VNS) signaling to a purchaser that the company selling the variety did not name it, and therefore probably did not develop it.

Branding is an issue that raises a number of controversial considerations revolving around the issue of multiple naming of a single variety. The basic logic for branding is that it may serve as an inducement to companies to market a specific variety. The negative aspects of a variety being marketed under more than one name must therefore be balanced with the positive aspects of greater potential public usage.

As stated above, there is a common misunderstanding in the field about Title V of the Federal Seed Act. Title V states that if the seed protected by a Plant Variety Protection Certificate is to be certified it must be sold by variety name. Many believe that if Title V is opted for when applying for a Plant Variety Certificate then branding is not allowed. However, this option does not rule out the possibility of branding, so long as seed is sold with the "variety not stated" designation.

LICENSING

The license agreement is "capstone" that can hold various negotiated terms together. The license itself is a grant to some portion of another's rights for a prescribed period of time. A license agreement is an extremely powerful instrument because it can accommodate differing economic, political and philosophical concerns of licenser and licensee. The license clauses can set forth the conditions of the relationship in as much detail as desired and can designate the law that will control the interpretation of those

clauses. Generally, the license will be enforced as long as it does not conflict with federal and state law and/or public policy.

A license agreement can include:

Ability to conduct research. There is some question as to whether or not utility patents extend the prohibition of "make, use or sell" to excluding others from doing research with the patentable subject matter. This is of course of critical concern to Universities, whose primary mission is to promote research. A doctrine has evolved over the years (called the "experimental purpose doctrine") whereby an accused infringer's making or using what is patented may be excused if it is solely for an experimental or other non-profit purpose. However, if research has a commercial end, then it could be an infringement. If the ability to conduct research is a concern in a specific situation, then the nature of the experimental use can be handled by a license agreement, wherein such use is either allowed or not allowed.

Access to germplasm. Another concern is that if germplasm is patented, then it will not be accessible to the general public. However, such allowances can be specified in a license agreement. One can specify whether or not access is *exclusive* or *non-exclusive*. If the seed is non-exclusive, then by definition, all can have access to the seed according to other terms of the agreement. If access is exclusive, the licensee may be made to make seed available to all who want it under acceptable sub-licensing terms.

Geographical limitations. It is important to realize that if one has filed patents world-wide, the license agreement can be used to specify where in the world the licensee(s) can use the invention. For example, if a patent has issued in both the United States and in Europe, one can still restrict a licensee to specific regions of the world. Furthermore, one may be able to specify that a royalty is to be collected in countries which do not have patent coverage.

Local Planting Politics. Pressure may be exerted from local constituencies to restrict commercial production to specific regions; license agreements again can specify this kind of item.

Royalty Distributions. One sometimes hears the objection to patenting or PVP because of opposition to royalty collection. Although this has nothing to do with the patent or PVP per se, the amount and distribution of royalties can be specified in the agreement--how much to the inventors, how much for research, etc.

Other documents which are becoming more common when handling plant intellectual property include *confidentiality agreements* and *research agreements*. Prior to the filing of patent applications, *confidentially agreements* can be executed with a potential licensee, simply acknowledging that the information to be shared is confidential and is not to be used for economic purposes. The sharing of seeds themselves can be preceded with *research agreements* that acknowledge that the seed is to be used for research purposes, and that intellectual property rights reside with the originator. For example, once patent applications are filed, then the content of the patent application can be shared with other researchers with little concern for losing one's competitive advantage, since the date of filing constitutes a legal priority date in most foreign countries. The contents of the patent application itself is published in most countries after 18 months, prior to the issuance of a patent.

CONCLUSION

In the future, all releases of plant varieties will be required to move through the same types of decision trees as we have presented here. Specifics may change from

country to country, but these basic elements will need to be considered. Obtaining intellectual property protection, negotiating and executing license agreements may increase transaction costs, but they reflect the reality and needs of today's marketplace.

REFERENCES

Federal Seed Act (1939), 7 U.S.C. 1551-1611, Et.Seq. (1988) (Title V can be found at § 1611).

Patent and Trademark Amendments Act (Bayh-Dole), P.L. 96-517 (1980). See 35 U.S.C.§§ 200-211 (1988).

Plant Variety Protection Act (1970), 7 U.S.C. 2321 Et Seq. 1988.

ADDITIONAL RELEVANT LITERATURE

Doyle, J. 1985. "Altered Harvest." Penguin, NY.

Hackleman, J.C. and Scott, W.C. 1990. "A History of Seed Certification in the United States and Canada." AOSCA, Raleigh, NC.

Juma, Çalestous. 1989. "The Gene Hunters: Biotechnology and the Scramble for seeds." Princeton University Press.

Kelly, A.F. 1989. "Seed Planning and Policy for Agricultural Production." Belhaven Press, London.

Kloppenburg, J.R. 1988. "First the Seed: The Political Economy of Plant Biotechnology, 1492-2000." Cambridge University Press, NY.

McDonald, M.B. and Pardee, W.D., eds. 1985. The Role of Seed Certification in the Seed Industry." CSSA. Madison, WI.

Stuber, C.W., Heichel, G.H. and Kissel, D.E., eds. 1989. "Intellectual Property Rights Associated with Plants." ASA Special Publication Number 52., Madison, WI.

NATURAL PRODUCT DRUG DISCOVERY AND DEVELOPMENT AT THE NATIONAL CANCER INSTITUTE: POLICIES FOR INTERNATIONAL COLLABORATION AND COMPENSATION

Gordon M. Cragg, Michael R. Boyd, Michael R. Grever, Thomas D. Mays[1], David J. Newman, and Saul A. Schepartz
Developmental Therapeutics Program, Division of Cancer Treatment
National Cancer Institute, Bethesda, Maryland 20892, USA

SUMMARY

An overview of the use of natural products in the treatment of cancer is presented as well as the current status of the NCI Natural Products Acquisition Program. Policies for International collaboration and compensation are discussed in reference to the NCI Letter of Intent. In addition, the distribution of extracts is discussed in context of the Material Transfer Agreement.

INTRODUCTION

Throughout the ages humans have relied on Nature as a source of medicines for the treatment of a variety of diseases. Plants, in particular, have been used in sophisticated traditional medicine systems for thousands of years in countries, such as China (Chang and But, 1986) and India (Kapoor, 1990), and there is archeological evidence of the use of medicinal plants in the New World tropics as early as 8,000 B.C. (King, 1992). According to World Health Organization statistics, these plant-based systems continue to play an essential role in the primary health care of 80% of the world's population (Farnsworth et al., 1985). The important role of plants in developed country health care systems is demonstrated by analysis of data on prescriptions dispensed from pharmacies in the United States from 1959 to 1980; about 25% of such prescriptions contained plant extracts or active principles from higher plants, and at least 119 chemical substances, derived from some 90 plant species, can be considered as important drugs currently in use in one or more countries (Farnsworth et al., 1985). Well-known examples of plant-derived medicinal agents include the antimalarial drug, quinine, isolated from the bark of *Cinchona officinalis*, the analgesics, codeine and morphine from *Papaver somniferum*, the tranquilizer, reserpine, from *Rauwolfia serpentina*, and the cardiac glycoside, digitoxin, from *Digitalis purpurea* (Farnsworth et al., 1985).

The role of microbial fermentation has been predominant in the development of antibiotics, with over 8,500 metabolites having been isolated from microbial sources, and close to 100,000 related agents having been prepared semisynthetically (Berdy, 1989). Well-known classes of antibiotics are the penicillins, cephalosporins, aminoglycosides,

[1]Office of Technology Development, National Cancer Institute, Bethesda, Maryland 20892, USA

and tetracyclines, while other microbial products include immunosuppressive, antiparasitic, and antifungal agents (Berdy, 1989). Few marine natural products of medicinal value have been developed to date, but recent investigations of marine organisms are yielding an increasing number of novel bioactive molecules (Faulkner, 1992; Pettit, 1991; Rinehart et al., 1990).

NATURAL PRODUCTS AND THE TREATMENT OF CANCER

Plants have a long history of use in the treatment of cancer (Hartwell, 1982), though many of the claims for the efficacy of such treatment might be viewed with skepticism, since cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine. The isolation of podophyllotoxin and other lignans exhibiting preclinical antitumor activity from *Podophyllum peltatum* in the 1950s (Jardine, 1979), and the discovery and development of the antileukemic agents, vinblastine and vincristine, from *Catharanthus roseus* (Carter and Livingston, 1976), however, provided convincing evidence that plants could be sources of a variety of novel, potential cancer chemotherapeutic agents. Recognition of this potential, and that of other natural materials, led the NCI in 1955 to initiate an extensive program for the procurement and screening of natural products for chemotherapeutic activity. Bioassay-guided fractionation of the active extracts resulted in the isolation and characterization of a large number of active agents belonging to a wide variety of chemical classes.

Between 1955 and 1980 screening of over 180,000 microbial extracts, mainly provided by the pharmaceutical industry, yielded a number of clinically useful anticancer agents, including doxorubicin (adriamycin), mitomycin C, bleomycin, and mithramycin, all derived mainly from *Streptomyces* strains (Boyd, 1993; Douros, 1976). Commercially available plant-derived anticancer drugs are the Vinca alkaloids, vinblastine and vincristine, and etoposide (O'Dwyer et al., 1985) and teniposide (O'Dwyer et al., 1984), semisynthetic derivatives of epipodophyllotoxin, an epimer of podophyllotoxin, while taxol, isolated from *Taxus brevifolia* and other *Taxus* species (Rowinsky et al., 1992), was approved for the treatment of advanced ovarian cancer in December, 1992. Taxol was discovered by the NCI through the screening of over 114,000 extracts derived from some 35,000 plant samples, collected by the United States Department of Agriculture (USDA) from mainly temperate regions in 60 countries; other promising agents in clinical development, and based on camptothecin which was discovered through this program, are topotecan (Kingsbury et al., 1991) and CPT-11 (Ohno et al., 1990), semisynthetic derivatives of camptothecin from *Camptotheca acuminata*. The thorough investigation of marine organisms as sources of anticancer agents only started in the mid-1970s. By 1981, over 16,000 extracts derived from over 500 species had been screened (Suffness and Douros, 1982), and two agents, didemnin B from *Trididemnum solidum* (Rinehart et al., 1990) and bryostatin 1 from *Bugula neritina* (Pettit, 1991), have advanced to clinical trials.

CURRENT STATUS OF THE NCI NATURAL PRODUCTS ACQUISITION PROGRAM

During 1985-1990, the replacement of the NCI's *in vivo* mouse leukemia primary screen by a new *in vitro* screening strategy, involving the use of 60 human cancer cell lines (Boyd, 1986, 1989, 1993), was paralleled by the implementation of new natural products acquisition, extraction, and isolation projects. The initiation, in 1987, of a

major new program within the NCI for the discovery and development of agents for the treatment of acquired immunodeficiency syndrome (AIDS) (Boyd, 1988) provided further impetus and resources for these projects.

Contracts for the collection of plants from tropical and subtropical regions of Africa and Madagascar, Central and South America, and Southeast Asia, were awarded to the Missouri Botanical Garden (MBG), the New York Botanical Garden (NYBG), and the University of Illinois at Chicago (UIC) (assisted by the Arnold Arboretum at Harvard University and the Bishop Museum in Honolulu), respectively, in 1986; these contracts were renewed for a further five years in 1991. Marine organism collections have centered on the Indo Pacific region since 1986, and collection contractors have included the Australian Institute of Marine Sciences (AIMS) and the Harbor Branch Oceanographic Institute, while the present contractor is the Coral Reef Research Foundation. Contracts for the cultivation of fungi, cyanobacteria, marine anaerobic bacteria, and protists, mainly obtained from established culture collections, have also been operating since 1985.

Plant and marine organism collections are being performed in over 25 mainly tropical, developing countries and, wherever possible, the NCI contractors work closely with qualified organizations in each of the source countries. Scientists from these organizations collaborate in field collection activities and taxonomic identifications, and make valuable contributions through their knowledge of local species and conditions. In addition, details of the medicinal uses and methods of preparation of plants by the local population or traditional healers are provided on a confidential basis when available. Such collaboration is supported through NCI contractor funding, and provides for expanded research activities by the local scientists; the NCI contractors also provide training opportunities for local personnel through conducting of workshops and presentation of lectures. Source country holdings of their natural resources are expanded through the deposit of a voucher specimen of each collected species in the national herbarium or repository. In turn, collaborating source country organizations provide facilities for the preparation, packaging, and shipment of samples to the NCI natural products repository in Frederick, Maryland, and the organizations' personnel have provided invaluable assistance to the NCI contractors in obtaining the necessary collection and export permits.

POLICIES FOR INTERNATIONAL COLLABORATION AND COMPENSATION. THE NCI LETTER OF INTENT

In carrying out these extensive collections and the investigation of natural products as potential sources of novel anticancer and anti-AIDS drugs, the NCI is very aware of the great value of the natural resources to each country participating in the collection programs, and of the significant contributions being made by source country scientists and, in some instances, traditional healers to the successful execution of these programs. Many of the participating countries have a real sensitivity to the possibility of exploitation of their natural resources by developed country organizations involved in drug discovery and other programs searching for novel bioactive agents (NAPRECA Newsletter, 1990).

The importance of these factors was emphasized shortly after award of the first contract by the NCI collection contractors, in particular those involved in plant

collections in developing nations, and led the NCI to formulate policies aimed at collaboration with, and compensation of, countries participating in the NCI drug discovery program. These policies are stated in the Letter of Intent (LOI) (see Appendix) which is provided to the NCI collection contractors for use in negotiations with country authorities for collection and export permits, and when establishing collaborations with source country organizations. In some instances, source country organizations and authorities have required the signing of a formal agreement based on the LOI prior to establishing collaborations and granting permits. As of March, 1993, agreements have been signed between the NCI, represented by its Director, and organizations in seven countries, including Cameroon, India, Madagascar, Philippines, Russia, Tanzania and Zimbabwe, while agreements are being considered with organizations in a number of other countries. In other cases, the NCI, on the recommendation of the collection contractors, has invited scientists from source country organizations to visit the drug discovery facilities at the Frederick Cancer Research and Development Center (FCRDC) to further discuss the goals of the program and the terms of the LOI, and to explore the scope for collaboration in the drug discovery effort. As of early 1993, 26 scientists from 18 countries had visited FCRDC and in some instances, U.S. contractor facilities, for periods of one to two weeks. These visits have invariably resulted in a better mutual understanding of the goals and concerns of each party, and have expedited the initiation of collection programs. In many cases, collection programs have been permitted to proceed without the signing of a formal agreement, but the NCI is always very willing to formalize an agreement at a later stage, should the source country desire it.

As stated in the introduction to the LOI, "while investigating the potential of natural products in drug discovery and development, the NCI wishes to promote the conservation of biological diversity, and recognizes the need to compensate source country organizations and peoples in the event of commercialization of a drug developed from an organism collected within their borders." The clauses contained in the LOI may be divided into short-term and long-term measures aimed at assuring source country organizations of the NCI's intentions to deal with them in a fair and equitable manner. In the short-term, the NCI, through its Developmental Therapeutics Program (DTP), undertakes to screen extracts of all organisms provided from the source country for anticancer and anti-AIDS activity, and to provide the test results to the source country organization on a confidential basis through the relevant collection contractor (clauses 1 and 2). Maintaining confidentiality of the results relating to active organisms is important, since the NCI may seek to patent a promising agent isolated from an active organism (clause 6), and disclosure of the test results prior to the patent application could invalidate the application, thereby jeopardizing the possibility for later compensation of the source country (clause 8). Further short-term measures include invitations to source country scientists to spend up to 12 months carrying out joint research projects with scientists in NCI facilities, providing suitable laboratory space and resources are available (clause 4), and an undertaking to test novel, pure compounds submitted by source country organization scientists for anticancer and anti-AIDS activity (clause 12). As of early 1993, 12 scientists from 9 countries have carried out, or are presently undertaking, collaborative research projects with scientists in NCI facilities.

Should an agent exhibit sufficient activity in the anticancer or anti-AIDS screens, it may be selected by the NCI for preclinical and clinical development. Such

development usually requires the procurement of large quantities of the source raw material for bulk production of the agent. Large-scale procurement could involve the sustainable harvest of the wild source organism, or might involve the development of a cultivation program. The NCI has developed contract mechanisms for the performance of such procurements, and will require the contractors to collaborate with the country of origin of the source organism in the provision of adequate supplies. In turn, the role of source country organizations as stated in the LOI includes an undertaking to collaborate with the NCI contractors in performance of such projects (clauses 3 and 4). In licensing a patent on a new drug to a pharmaceutical company, the NCI will require the licensee to seek, as its first source of supply, the raw material produced in the country of origin, subject to negotiation of a mutually agreeable fair price (clause 10).

Should an agent exhibit sufficient promise for development as a potential novel drug, the NCI will seek patent protection on the agent (clause 6) and may license the agent to a pharmaceutical company for production and marketing (clause 8). In such an event, the NCI undertakes to "make its best effort to ensure that royalties and other forms of compensation shall be provided to the host (source) country organization and to individuals of that country, as appropriate, in an amount to be negotiated with the NCI in consultation with the host (source) country organization" (clause 8). These terms would apply equally to instances where the agent is the actual isolated natural product, or where it is a product structurally based on the isolated natural product (clause 9). By U.S. law, the NCI cannot be obligated to a given level of royalties in the LOI or any agreement based thereon, since as a U.S. Government Agency, it is not authorized to promise or encumber **future** intellectual property (patent) rights to any invention of the NCI, except under the terms of a cooperative research and development agreement (CRADA) [15 U.S.C. Sec. 3710 (a) (b) (2) (1991)]. Generally CRADAs are only considered in the case of projects which are in a state of advanced research and development. It is also the policy of the National Institutes of Health (NIH) Patent Policy Board to defer negotiations and agreement upon specific royalty rates until the specific invention is ascertained. Royalty rates can vary considerably depending on the potential applications and value of the invention. In addition, U.S. law forbids any attempt by a source country to restrict the U.S. Government's ability to license or assign its intellectual property [35 U.S.C. Sections 207, 208 and 209]. It must be stressed, however, that use of the term "make best effort" has a particular meaning in U.S. law in that it implies a strong commitment on the part of the NCI to pursue a policy of fair and equitable compensation. Since eventual development of a drug to the stage of marketing may require 10 to 15 years, compensation must necessarily be regarded as a long-term measure.

POLICIES FOR DISTRIBUTION OF EXTRACTS TO ORGANIZATIONS OUTSIDE THE NCI MATERIAL TRANSFER AGREEMENTS

Considerable interest has been shown by other research organizations and companies in the screening of materials from the NCI Natural Products Repository (NPR) for activities against diseases related to the NCI mission, including diseases which have an impact on health in developing countries. After consulting source country representatives, either directly or through their respective collection contractors, the NCI has formulated a policy for the distribution of extracts to carefully selected organizations

(clause 13). In applying for access to NPR materials, research organizations and individual investigators must submit research proposals which clearly delineate the research to be performed. The importance and relevance to the NCI mission, prior work in the area, the specific approach to be used, and the ability to carry out the research in a timely manner all need to be clearly defined in the proposals. Proposals are reviewed by a committee of senior NCI staff, and are judged primarily on their scientific merit and the relative importance of the proposed research. A key factor determining the selection of recipient organizations is the agreement by the organizations to abide by the same policies of compensation and raw material supply as stated in the LOI (clauses 8, 9 and 10). Approval for distribution of extracts is granted only after the organization has signed a legally binding material transfer agreement with the NCI. The material transfer agreement contains the following clause guaranteeing the rights of source countries to compensation in the event of production and marketing of a drug from an organism collected within their borders; this clause also requires the recipient to utilize the host (source) country as the first source of supply of raw material for production of the marketed agent:

"Recipient acknowledges that NCI has obtained this material from the host country under an agreement that provides for the payment of a royalty fee to be negotiated in good faith should the material result in an agent that is further developed. In order to comply with this obligation and to protect the interests of the country of origin and the contributors of the Research Material, recipient further agrees that, should an agent eventually be produced and marketed, royalties and other forms of compensation shall be provided to the host country organization and to individuals of that country, as appropriate, in an amount to be negotiated with NCI in consultation with the host country organization. Such terms shall apply equally to instances where the invention is the actual isolated natural product, or where the invention is a product structurally based on the isolated natural product (i.e., where the natural product provides the lead for development of the invention), though the royalty rates and other compensation negotiated as payment might vary depending on the nature of the drug being licensed, the relative research and development effort required, and the royalty rates conventionally granted in the relevant field of use. Recipient further agrees to utilize the host country as its first source of supply of the raw material for the marketed product, or an agent derived therefrom, if such material can be made available. If such material must be cultivated, recipient agrees to seek to utilize the host country as its first source of such cultivation efforts."

The expansion of the screening of extracts beyond the NCI anticancer and anti-AIDS test systems will enhance the chances for the discovery of new drugs, and thereby enhance the possibility for compensation of countries participating in the NCI collection programs.

CONCLUSION

The NCI has formulated the Letter of Intent in response to the legitimate concerns of countries participating in collection programs that they receive fair and equitable treatment in return for investigation of their valuable natural resources. The policies developed by the NCI includes measures aimed at collaboration in the short term, and compensation in the long term for any commercial drugs developed through the program. Some source countries have required the signing of formal agreements

based on the LOI, while others have permitted collections to proceed without establishing such agreements. Irrespective of the existence or absence of formal agreements, the NCI is strongly committed to the policies of collaboration with, and compensation of, source countries as stated in the LOI. These policies have received full support from all levels of the NCI and the National Institutes of Health, and have elicited support from members of the U.S. Congress and various non-governmental organizations. Collaborations have also been established with research organizations in countries not covered by the present collection contracts, or organizations studying organisms not included in the NCI program. In establishing such collaborations, the NCI undertakes to abide by the same policies of collaboration and compensation as stated in the LOI, and, in some instances, has entered into agreements based on the LOI.

Any outside research organizations approved by the NCI for access to materials from the NCI natural products repository are legally bound to the same policies of compensation and raw material supply as stated in the LOI.

ACKNOWLEDGEMENTS

The NCI gratefully acknowledges the collaboration and support of the many individuals and organizations worldwide which make these programs possible. From the collection of organisms in over 25 countries to the clinical trials of new drugs, and the studies of occurrence and prevention, this is truly an international effort in the fight against the scourges of AIDS and cancer. The NCI recognizes the indispensable contributions being made through the provision of valuable natural resources, expertise, knowledge, and skills; through policies of collaboration and compensation, as stated in the Letter of Intent, the NCI wishes to assure participating countries of its intentions to deal with them in a fair and equitable manner.

REFERENCES

Berdy, J. 1989. The discovery of new antibiotic microbial metabolites: Screening and identification. Prog. in Microbiol. 27:3-25.

Boyd, M.R. 1986. National Cancer Institute Drug Discovery and Development, *in*: "Accomplishments in Oncology Vol. 1, No. 1. Cancer Therapy: Where Do we Go From Here?" E.J. Frei and E.J. Freireich, eds., Lippincott, Philadelphia.

Boyd, M.R. 1988. Strategies for the identification of new agents for the treatment of AIDS: A national program to facilitate the discovery and preclinical development of new drug candidates for clinical evaluation, *in:* "AIDS: Etiology, Diagnosis, Treatment, and Prevention," V.T. Devita, S. Hellman and S.A. Rosenberg, eds., J.B. Lippincott, Philadelphia.

Boyd, M.R. 1989. Status of the NCI preclinical antitumor drug discovery screen, *in*: "Principles and Practice of Oncology Updates, Vol. 3, No. 10.," V.T. Devita, S. Hellman, and S.A. Rosenberg, eds., J.B. Lippincott, Philadelphia.

Boyd, M.R. 1993. The future of new drug development, *in*: "Current Therapy in Oncology. Section 1. Introduction to Cancer Therapy," J. Niederhuber, ed., B.C. Decker, Inc., Philadelphia.

Carter, S.K. and Livingston, R.B. 1976. Plant products in cancer chemotherapy. Cancer Treatment Rep. 60:1141-1156.

Chang, H-M. and But, P.P-H. 1986. "Pharmacology and Applications of Chinese Materia Medica, Vols. 1 and 2." World Scientific Publishing Co., Singapore.

Douros, J.D. 1976. Lower plants as a source of anticancer drugs. Cancer Treat. Reports, 60:1069-1080.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D. and Guo, Z. 1985. Medicinal plants in therapy. Bulletin of the World Health Organization 63:965-981.

Faulkner, D.J. 1992. Biomedical uses of natural marine chemicals. Oceanus 29-35.

Hartwell, J.L. 1982. "Plants Used Against Cancer. A Survey," Quarterman Publications, Inc., Lawrence, Massachusetts.

Jardine, I. 1979. Podophyllotoxins, *in*: "Anticancer Agents Based on Natural Product Models," J.M. Cassady and J.D. Douros, eds., Academic Press, New York.

Kapoor, L.D. 1990. "CRC Handbook of Ayurvedic Medicinal Plants," CRC Press Inc., Boca Raton, Florida.

King, S.R. 1992. Conservation and tropical medicinal plant research. Herbalgram 27:28-35.

Kingsbury, W.D., Boehm, J.C., Jakas, D.R., Holden, K.H., Hecht, S.M., Gallagher, G., Caranfa, M.J., McCabe, F.L., Faucette, L.F., Johnson, R.K. and Hertzberg, R.P. 1991. Synthesis of water-soluble (aminoalkyl) camptothecin analogues: Inhibition of topoisomerase I and antitumor activity. J. Med. Chem., 34:98-107.

NAPRECA Newsletter. 1990. Researchers want clear policies on traditional herbs. International Conference of Experts of Developing Countries on Traditional Medicinal Plants, Arusha, Tanzania, 7:2.

O'Dwyer, P., Alonso, M.T., Leyland-Jones, B. and Marsoni, S. 1984. Teniposide. A review of 12 years of experience. Cancer Treat. Rep. 68:1455-1466.

O'Dwyer, P., Leyland-Jones, B., Alonso, M.T., Marsoni, S. and Wittes, R.E. 1985. Etoposide (VP-16-213). Current status of an active anticancer drug. New Engl. J. Med. 312:692-700.

Ohno, R., Okada, K., Masaoka, T., Kuramoto, A., Arima, T., Yoshida, Y., Ariyashi, H., Ichimaru, M., Sakai, Y., Oguro, M., Ito, Y. Morishima, Y., Yokomaku, S. and Ota, K. 1990. An early phase II study of CPT-11: A new derivative of camptothecin, for the treatment of leukemia and lymphoma. J. Clin. Oncol. 8:1907-1912.

Pettit, G.R. 1991. The Bryostatins, *in:* "Progress in the Chemistry of Organic Natural Products," W. Herz, G.W. Kirby, W. Steglich, and C. Tamm, eds., Springer-Verlag, New York.

Rinehart, K.L., Holt, T.G., Fregeau, N.L., Keifer, P.A., Wilson, G.R., Perun, T.J., Jr., Sakai, R., Thompson, A.G., Stroh, J.G., Shield, L.S., Seigler, D.S., Li, L.H., Martin, D.G., Grimmelikhuijzen, C.J.P. and Gade, G. 1990. Bioactive compounds from aquatic and terrestrial sources. J. Nat. Prod. 53:771-792.

Rowinsky, E.K., Onetto, N., Canetta, R.M. and Arbuck, S.G. 1992. Taxol: The first of the Taxanes, an important new class of antitumor agents. Seminars in Oncol. 19:646-662.

Suffness, M. and Douros, J. 1982. Current status of the NCI plant and animal product program. J. Nat. Prod. 45:1-14.

APPENDIX

The NCI Letter of Intent

The Developmental Therapeutics Program (DTP), Division of Cancer Treatment (DCT), National Cancer Institute (NCI) is currently investigating plants, marine macro-organisms and microbes as potential sources of novel anticancer and AIDS-antiviral drugs. The DTP is the drug discovery program of NCI which is an Institute of the National Institutes of Health supported by the United States Government. While investigating the potential of natural products in drug discovery and development, NCI wishes to promote the conservation of biological diversity, and recognizes the need to compensate source country organizations and peoples in the event of commercialization of a drug developed from an organism collected within their borders.

As part of the drug discovery program, DTP has contracts with various organizations for the collection of plants and marine macro-organisms worldwide. DTP has an interest in investigating [*name of organisms*] from [*name of country*], and wishes to collaborate with [*country organization*] in this investigation. Such a collaboration will be within the framework of the collection contract between NCI and [*collection contractor*].

The role of DTP, DCT, NCI in the collaboration will include the following:

1) DTP will screen the extracts of all [*name of organisms*] provided from [*country*] for anticancer and AIDS-antiviral activity, and will provide the test results to [*country organization*] as soon as they are available. Such results will be channeled via [*collection contractor*].

2) The test results will be *kept confidential by all parties*, with any publication delayed until DTP has an opportunity to file a patent application in the United States of America on any active agents isolated.

3) Any extracts exhibiting significant activity will be further studied by bioassay-guided fractionation in order to isolate the pure compound(s) responsible for the observed activity. Since the relevant bioassays are only available at DTP/NCI, such fractionation will be carried out in DTP/NCI laboratories.

4) Subject to the provision that suitable laboratory space and other necessary resources are available, DTP/NCI agrees to invite a senior technician or scientist designated by [*country organization*] to work in the laboratories of DTP/NCI or, if the parties agree, in laboratories using technology which would be useful in furthering work under this agreement. The duration of such a visit would not exceed one year except by prior agreement between [*country organization*] and DTP/NCI. The designated Guest Researcher will be subject to provisions usually governing Guest Researchers at NIH. Salary and other conditions of exchange will be negotiated in good faith.

5) In the event of the isolation of a promising agent from a [*name of organism*] collected in [*country*], further development of the agent will be undertaken by DTP/NCI.

6) DTP/NCI will, as appropriate, seek patent protection on all inventions developed under this agreement by NCI employees alone or by NCI and [*country organization*] employees jointly, and will seek appropriate protection abroad.

7) All licenses granted on any patents arising from this collaboration shall contain a clause referring to this agreement and shall indicate that the licensee has been apprised of this agreement.

8) Should the agent eventually be licensed to a pharmaceutical company for production and marketing, DTP/NCI will make its best effort to ensure that royalties and other forms of compensation shall be provided to the host country organization and to individuals of that country, as appropriate, in an amount to be negotiated with NCI in consultation with the host country organization.

9) Such terms shall apply equally to instances where the invention is the actual isolated natural product, or where the invention is a product structurally based on the isolated natural product (i.e., where the natural product provides the lead for development of the invention), though the percentage of royalties negotiated as payment might vary depending upon the relationship of the marketed drug to the originally isolated natural product.
It is understood that the eventual development of a drug to the stage of marketing is a long-term process which may require 10-15 years.

10) In obtaining licensees, the DTP/NCI will require the applicant for license to seek as its first source of supply the natural products available from [*country*]. If no appropriate licensee is found who will use natural products available from [*country*], or if [*country organization*] or its suppliers cannot provide adequate amounts of raw materials at a mutually agreeable fair price, the licensee will be required to pay to [*country organization*] an amount of money (to be negotiated) to be used for expenses associated with cultivation of medicinal plant species that are endangered by deforestation, or for other appropriate conservation measures.

11) Sections 7-10 shall not apply to organisms which are freely available from different countries (i.e., common weeds, agricultural crops, ornamental plants, fouling organisms) unless information indicating a particular use of the organism (e.g., medicinal, pesticidal) was provided by local residents to guide the collection of such an organism from their country, or unless other justification acceptable to both the [*country organization*] and

DTP/NCI is provided. In the case where an organism is freely available from different countries, but a genotype producing an active agent is found only in [*name of country*], sections 7-10 shall apply.

12) DTP/NCI will test any pure compounds submitted by [*country organization*] scientists for antitumor and AIDS-antiviral activity, provided such compounds have not been tested previously in the NCI screens. If significant antitumor or AIDS-antiviral activity is detected, further development of the compound and investigation of patent rights will, as appropriate, be undertaken by DTP/NCI in consultation with [*country organization*].

Should the agent eventually be licensed to a pharmaceutical company for production and marketing, DTP/NCI will make its best effort to ensure that royalties and other forms of compensation shall be provided to the host country organization and to individuals of that country, as appropriate, in an amount to be negotiated with NCI in consultation with the host country organization.

13) DTP/NCI may send selected samples to other organizations for investigation of their anti-cancer, anti-HIV or other therapeutic potential. Such samples will be restricted to those collected by NCI contractors unless specifically authorized by the country organization. Any organization receiving samples must agree to compensate the source country organization and individuals, as appropriate, in the same fashion as is described in sections 8-10 above, notwithstanding anything to the contrary in section 11.

The role of [*country organization*] in the collaboration will include the following:

1) [*Country organization*] will collaborate with [*collection contractor*] in the collection of [*name of organisms*], and will work with [*collection contractor]* to arrange the necessary permits to ensure the timely collection and export of materials to DTP/NCI.

2) Should [*country organization*] have any knowledge of the medicinal use of any [*name of organisms*] by the local population or traditional healers, this information will be used to guide the collection of such organisms on a priority basis where possible. Details of the methods of administration (e.g., hot infusion, etc.) used by the traditional healers will be provided where applicable to enable suitable extracts to be made. All such information will be kept confidential by DTP until both parties agree to publication.

The permission of the traditional healer or community will be sought before publication of their information, and proper acknowledgment will

be made of their contribution.

3) [*Country organization*] and [*collection contractor*] will collaborate in the provision of further quantities of active raw material if required for development studies.

4) In the event of large amounts of raw material being required for production, [*country organization*] and [*collection contractor*] will investigate the mass propagation of the material in [*country*]. Consideration should also be given to sustainable harvest of the material while conserving the biological diversity of the region, and involvement of the local population in the planning and implementation stages.

5) [*Country organization*] scientists and their collaborators may screen additional samples of the same raw materials for other biological activities and develop them for such purposes independently of this agreement.

	Name (Signature)
	Name (Print or type)
	Title
	Institution or Agency
Director, National Cancer Institute	Address
Date	Date

BIOTECHNOLOGY IN DEVELOPING COUNTRIES: PARTICIPATION BY PRIVATE INDUSTRY

Robert B. Horsch
Monsanto Company, 700 Chesterfield Village Parkway
St. Louis, Missouri 63198, USA

SUMMARY

Private companies have three methods of protecting their investments in new crops: Hybrids; plant variety protection/plant patents; and patented genes. The latter two methods depend on international laws and agreements. The role of private industry in transferring biotechnology to developing countries is examined by way of illustration using a joint Monsanto - USAID - Kenya project on sweet potato virus as a model. Three factors are identified for a successful technology transfer to developing countries: an important solvable problem; a design for a program to work towards solving that problem and to transfer the technology at the same time; and finding a person(s) and an institution with the capacity to carry the project to completion.

INTRODUCTION

Single genes can confer desirable and valuable traits to plants and to the harvest of food or fiber from those plants. Benefits derived from these genes will include vine-ripened tomatoes, crisper, less oily chips or French fries, and more abundant harvests from crops that are better protected from destructive insects or diseases. These single gene traits will be combined with the best new varieties produced by traditional plant breeding and will accelerate the pace and the scope of our ability to develop ever better and more productive crops. An excellent review of progress with transgenic crops can be found in a recent article by Gasser and Fraley (1992).

These genetically engineered improvements are not dramatically different than the types of improvements developed by plant breeders since mankind first began to deliberately plant seeds from one plant instead of another because of some beneficial characteristic. During this century, plant breeders have been particularly effective at cross-breeding exotic genes from wild species into cultivated varieties of related crops. For the most part, these new genes cause a measurable change in one or more properties of the plant, such as disease resistance, stature, stress tolerance, seed quality, nutritional properties etc. However, in nearly every case, the mode-of-action of the gene is unknown to the breeder.

The effective gene pool potentially available to plant breeders is now larger through the use of gene cloning, gene splicing, and gene transfer technologies. For the most part, finding and using these genes begins with knowledge about their mode of action or with a screen for specific biological activity. One of the most important sources of beneficial genes for crop improvement is the vast gene pool contained in the many, diverse species of plants and other organisms that live and grow around the world today. Our capacity to study, to understand, and to find the most useful new genes from nature is still very limited, but growing steadily each year. Even as we are just beginning to tap this huge and valuable resource, it is being destroyed by loss of natural habitat and environmental destruction. The terrible irony is that effective use of the biodiversity on earth is one of the single most powerful means to protect it - if we act before it is lost.

The products of biotechnology will provide major gains in agricultural productivity and effective biological means to prevent and to remediate pollution. The more productive we can be with good crop land, the less natural habitat (which is usually poor farm land anyway) will need to be cleared and plowed for growing food.

INVESTMENT IN BIOTECHNOLOGY RESEARCH

Both public and private investments have been made in plant breeding programs, resulting in benefits to society far in excess of the original investments. While most of the value derived from these research investments have been essentially public domain from the beginning, there are several types of mechanisms in the US that have been used to protect new varieties and provide a profit to the investors. The plant variety protection act and plant patents provide for an exclusive period of protection for precisely described plant varieties during which no one can propagate that exact variety. A second mechanism is hybridization where the hybrid seed must be purchased from the producer each year since the progeny seed will not breed true and will lose the desirable properties of the hybrid. A third, very recent mechanism that addresses genes and therefore plants containing patented genes, is the US patent system which provides 17 years of protection for patented genes or processes used during product development. Since many developing countries do not offer patent protection for genes or plant varieties, only hybrid seeds provide a means to protect investments made in developing new varieties. In the absence of hybrids, there is little incentive for private investment in development of new varieties that are locally adapted or even to introduce successful varieties from elsewhere in the hope that they will perform well. In this case, public institutions have provided new varieties, sometimes with remarkable success and sometimes not.

The patent system is an ingenious mechanism to provide for stimulation of private investment and entrepreneurial enterprise while fostering scientific communication and technological progress among competing inventors or institutions. It does this by granting a defined period of exclusive ownership for inventions but requires enabling public disclosure of how to practice the invention at issuance of the patent. This public disclosure allows all other researchers and inventors in the field to use the invention and associated knowledge to improve on the invention and create inventions of their own. Since the invention is disclosed to the public, once the patent expires, the invention enters the public domain for future generations to use.

The initial wave of genetic improvements such as insect, virus and herbicide resistance were first field tested in 1987 and are still several years away from commercial release. Some of the key hurdles from discovery of a promising gene to a commercial product are: i) gene improvement and vector construction, ii) product development, iii) field testing, iv) breeding into multiple elite varieties, v) product characterization and regulatory review, vi) public acceptance, and vii) marketing. Each step is expensive, uncertain and time consuming. The profit made during the product's commercial life must pay for the direct expenses of its development and marketing, cover the costs of the leads that failed at some point, and provide more return than putting the same capital into the bank or other investments.

Insect resistant cotton is a good example of the time and effort involved. The Bt genes for insect resistance have been known to exist for more than 30 years, and were first isolated in 1982. It took 4 or 5 generations of gene improvement from 1984 to 1990 to produce a version of the gene that would adequately express in plants. It will

take another 6 years to introgress the best gene into several commercial cultivars of cotton and to bulk up the volume of improved seed available before the first commercial sales would be possible. Concurrently, three years of multi-location field testing will be needed to ensure product efficacy under a wide range of environments and geographic regions. Also concurrently, product characterization and regulatory data submission, review and approvals will take between 3 and 5 or more years. Then, if everything is still satisfactory, the product can be released for sale and compete against existing products and technology for insect control.

TECHNOLOGY TRANSFER TO DEVELOPING COUNTRIES

A number of public and private foundations and institutions have targeted biotechnology as a key technology for solving food production problems in developing countries. The Rockefeller Foundation has funded a major program in Rice Biotechnology. The World Bank, USAID, UNESCO, ORSTOM and others have also launched large funding programs to build institutional capacity for biotechnologies around the world. They have recognized the natural applicability of this technology for use by resource poor farmers since the product comes packaged in a seed. Persley (1990) provides a good discussion of the fit of biotechnology with international institutions, programs and needs.

Several private companies are also involved in technology transfer projects. One example was begun during 1990, when initial discussions were held between Monsanto Company and USAID about possible ways that private companies could help with technology transfer to developing countries in the agricultural biotechnology field. These discussions led to a proposal which was jointly funded by USAID and Monsanto. The first objective was to find a serious problem that was technically feasible to solve with current technology. The second was to design a program to work towards solving that problem and to transfer the technology at the same time. The third was to find a person and an institution with the capacity to carry the project to completion after the training program.

AN IMPORTANT, SOLVABLE PROBLEM

In many developing countries, losses of the root and tuber crops due to viral infection can reduce potential production by 20-80% depending on the country. In collaboration with Dr. Roger Beachy, Monsanto Company and Washington University had developed a proprietary technology to create transgenic plants that were viral disease resistant . Through genetic engineering of the coat protein gene of the virus, using proprietary Monsanto technology for the production of such plants, it was possible to create viral disease resistant plants. The key components of the technology are coat protein gene clone, gene expression construction, transformation vector, and a transformation, selection and regeneration process. Downstream technologies include assays for presence of the virus and for the coat protein, as well as field testing capacity. It seemed reasonable that the best way to train a person in these technologies was to have them actually engage in doing the project.

A GOOD PARTNER AND RECIPIENT INSTITUTION

The specific choice of crop and virus was deliberately left open while recruiting a project participant so that the final choice could be made in consultation with the African partner institution. With help from USAID, Dr. Beachy and others, an excellent partner

was recruited for this program, Florence Wambugu from the Kenyan Agricultural Research Institute. Wambugu had studied sweet potato viruses in the field throughout east Africa and had become one of the leading experts on this problem (Wambugu, 1991). She also had just completed studies on sweet potato regeneration from tissue culture - a key prerequisite to transformation. She was therefore doubly qualified for the project. Not only was Dr. Wambugu personally committed to improving sweet potatoes, but she had the support of her home institution, KARI.

While SPFMV is probably the most wide-spread sweet potato virus, it does not cause major losses by itself. However, it is frequently found in combination with one of several other viruses, resulting in a synergistic increase in disease severity. SPFMV is thereby implicated in many of the most devastating viral diseases of sweet potato. By controlling this one virus, it is expected that many serious double virus diseases will be controlled.

OBJECTIVES OF THE PROJECT

The objectives of this project are to begin the development of virus resistant sweet potatoes and to transfer the technology to an African institution. The training Dr. Wambugu participates in includes: (1) basic training in plant cell and tissue culture as well as in plant molecular biology, (2) research to improve regeneration technology for sweet potato, with emphasis on African varieties, (3) development of *Agrobacterium* transformation techniques for sweet potato tissues, (4) development of selection parameters for kanamycin resistance, (5) coupling the regeneration techniques with transformation, (6) production of transgenic sweet potatoes with SPFMV vectors. Associated training includes assays to monitor the genes and virus resistance as well as knowledge of the regulatory science and process in the US.

INNOVATIVE ASPECTS

This proposal presents a unique opportunity to learn the issues that are important in technology transfer, as well as to accelerate the rate of technology transfer to less developed countries which have limited resources to develop their own technologies for improving agriculture using modern biological knowledge and tools. This model experiment, when successful, could provide an enormous stimulus for accelerating the use of the technology which does not require complex capital intensive tools to implement, once the basic regeneration and transformation technologies are developed, and the gene technologies have been perfected.

In this regard, a critical and innovative aspect of this proposal consists of the willingness of Monsanto to provide proprietary technologies for coat protein mediated virus resistance in transgenic sweet potato for use within Africa. Second, it is recognized that substantial expertise has been gained by Monsanto in regard to conducting field tests in compliance with U.S. regulatory agency rules and guidelines. Monsanto has conducted more than 300 field tests on 6 crops over the last five years. Monsanto scientists can provide consultation based on this experience to USAID and Kenyan government officials to facilitate the establishment of appropriate guidelines for the anticipated field tests in Kenya.

A second, possibly larger program will be needed to ensure that appropriate biosafety guidelines are developed in conjunction with Kenyan government officials. It will be necessary to develop a program to document the food safety of the improved virus resistant sweet potato before introduction into food uses in Africa. Since no food

products have yet been marketed in any country, the extent of required testing is not known at this time. While no food safety issues can be foreseen that would hinder the adaptation of this new technology, prudence and public acceptance will certainly dictate a significant degree of product testing. Monsanto can provide consultation on food safety issues to USAID, KARI and Kenyan government officials. A number of international public institutions are also available to provide information and consulting on issues related to food safety and environmental and environmental safety of biotechnology processes and products.

OTHER PUBLIC PRIVATE COLLABORATIONS

A recent program launched by USAID at Michigan State University has incorporated programs involving companies such as DNAP (Sondahl, 1993) and ICI Seeds (Wilson, 1993) with public institutions in Indonesia and Costa Rica. A new non-profit foundation headquartered at Cornell University, ISAAA, the International Service for the Acquisition of Agribiotech Applications, has brokered programs between Monsanto Company and CINVESTAV in Mexico to develop local potato varieties that are resistant to PVX and PVY, and is actively pursuing a variety of similar projects with other institutions.

CONCLUSION

As products derived from biotechnology research approach the market place in developed and developing countries, the issues raised by intellectual property rights, or lack thereof, will become more important. A new paradigm is needed for ensuring the maximum benefits from this new and powerful field of science. In the long run all countries will benefit from clear, enforceable protection for intellectual properties such as improved genes and transgenic crops. This will provide incentive for private investment in the most productive sectors of agriculture by multinational companies and for local entrepreneurial companies alike, providing products, food, income and tax revenues for the country. This will free the public institutions to invest more in long-range research, orphan crops, education, and extension programs.

REFERENCES

Gasser, C.S. and Fraley, R.T. 1992. Transgenic crops. Scientific American June 62-69.

Persley, G.J. 1990. Beyond Mendel's Garden: Biotechnology in the Service of World Agriculture. C A B International, Wallingford.

Sondahl, M. 1993. *in:* BioLink Volume 1, Number 2, Newsletter of the Agricultural Biotechnology for Sustainable Productivity Project, Michigan State University, East Lansing.

Wambugu, F. 1991. Ph.D dissertation, *In vitro* & Epidemiological Studies of Sweet Potato Virus Diseases in Kenya.

Wilson, M. 1993. *in:* BioLink Volume 1, Number 2, Newsletter of the Agricultural Biotechnology for Sustainable Productivity Project, Michigan State University, East Lansing.

MODELS FOR ETHICAL COLLABORATION IN BIODIVERSITY PROSPECTING

James S. Miller and Daniel K. Harder
Missouri Botanical Garden, P.O. Box 299
St. Louis, Missouri 63166-0299, USA

SUMMARY

The ethics of various kinds of collaborations are examined through discussions of intellectual property rights, ownership of national biodiversity and current models of collaboration.

INTRODUCTION

In 1991, the American Bar Association began project ABIGAILE (American Bar Intergenerational Accords on the International Law of the Environment), an effort to review international environmental law sources and produce statements of policy (Paul, 1991). The topics of primary focus included climate change, transnational environmental damage, deforestation, ozone destruction, soil loss, and biodiversity. The position adopted on biodiversity basically refused to accept that the total biological diversity of a given country was a sovereign resource exclusively of that country, but should be viewed as a resource of all humanity. In opposition to the majority of the nations of the world, this position was adopted by the United States Government at the United Nations Earth Summit, held in Brazil in 1992, where this country refused to sign the Convention on Biological Diversity.

This brought greatly increased attention to an already ongoing discussion of Sovereign and Intellectual Property Rights as they relate to international transport of biodiversity for commercial exploitation. Discussions of such diverse issues as protection of novel plant varieties, drug development from natural products, and intellectual property rights of indigenous healers were already underway in a wide variety of formats. All of these topics have received considerable debate recently as various groups from diverse backgrounds have struggled towards a consensus.

Recent discussions of the ethical considerations that are related to biodiversity prospecting have included diverse topics, far beyond what is traditionally implied by the term intellectual property rights. Lately, the term has been used to refer to any ethical question of any aspect of programs to research commercial exploitation of biodiversity, rather than being restricted to questions of true Intellectual Property.

INTELLECTUAL PROPERTY RIGHTS

It is necessary to understand the diversity and classification of ethical issues that are relevant to these programs before considering the options that are possible for dealing with them. While the topic of Intellectual Property Rights has been used in a very broad sense, sometimes included with or loosely associated with issues of training, institution building, and information transfer, a narrower definition is more precise and the remaining issues should be relegated to other areas of ethics. Thus, the definition of Intellectual Property Rights is best restricted to those rights that protect products of our intellect. This would follow the definition of intellectual property rights from Price (1992), "an aggregate of rights resulting from the creative efforts of the mind." Thus an

innovative collaborative program would contain mechanisms for protecting Intellectual Property Rights and Sovereign Rights, which can be legally protected, but should also deal with equity in research opportunities and professional and institutional development (Table 1).

Table 1. Areas of Ethical Questions in Biodiversity Prospecting.

Intellectual Property Rights
Trade Secrets
Patents
Sovereign Rights
Research Opportunities
Professional and Institutional Development

Intellectual property can be classified as industrial property, generally inventions or industrial designs that can be protected by patents or trademarks, or other property, generally from the literary, musical, or visual arts that can be protected by copyright (Anonymous, 1993). There are many legal methods for the protection of intellectual property including trademarks, copyrights, trade secrets, and patents, but the latter two are most relevant to this discussion. Information from traditional healers, including those plants which are useful and the proper method of preparation for administration, might be considered trade secrets and thus subject to legal protection (Gollin, 1993). Information can be considered a trade secret if it is confidential and provides an advantage over competitors, is secret, and is subject to efforts to maintain its secrecy. The United States Supreme Court has upheld that disclosure of trade secrets is taking of property, and thus subject to just compensation (Gollin, 1992). Tubocurarine was discovered from *Chondrodendron tomentosum*, commonly used as a source of arrow poison in South America. However, it is unlikely that courts would consider this protected as a trade secret as the information regarding its use was generally available and not subject to secrecy. On the other hand, many of the herbal remedies of healers are in fact carefully guarded information that could legally be considered to be trade secrets, and therefore subject to compensation in the case of disclosure. As the collection and study of this type of information is the central interest of ethnobotanical research, many of the guidelines for interaction with traditional healers have come from resolutions, codes of ethics, and guidelines of various ethnobotanical societies. The Society for Economic Botany (Paddoch and Boom, 1991) produced a draft code of ethics that included ethical recommendations for collecting and disseminating information from traditional healers as well as providing means of compensation when information legally classifiable as trade secrets is divulged through publication or other means. Thus, the draft code of ethics, not specifically designed to be relevant in the context of a drug discovery program, did address issues that could be trade secrets or patentable, and also related issues of information transfer and professional and institutional development.

Successful discoveries, breakthroughs, or innovations in a drug discovery program can also be considered intellectual property and if they meet a series of requirements, are patentable. In order to be patentable, an item or process must be a new invention, must be useful, and not an obvious extension of what is already in the public domain (Price, 1992). Patents provide for the exclusive use of the item or process for a period of time by the holder of the patent or licensee.

RIGHTS TO BIODIVERSITY

Whether countries have an exclusive right to their biodiversity as a resource for potential commercial development and whether these countries deserve a return of royalties, or other just compensation or profit sharing, for products that result from exploitation of their biodiversity is another question worth exploring. These sovereign property rights are clearly separate and distinct from intellectual property rights. This was the major point addressed by the Convention on Biological Diversity and why the United States refused to sign during the United Nations Earth Summit in Brazil. While a wide variety of arguments have been put forward regarding this issue, the reality is that most developing countries are demanding some guarantee of a share of the profits from products that are developed from their plants, animals, and microorganisms. Organizations that fail to provide viable mechanisms to accomplish this will simply be denied permits for collection and exportation. Issues that should be considered under sovereign rights would include compensation, profit sharing, and transfer of information generated by research.

DEVELOPMENT OF A PLANT DERIVED DRUG

The development of a drug from natural sources generally takes between ten and fifteen years with an average cost that had been estimated at 231 million dollars in 1990 but is probably between 285 and 300 million dollars in 1993 (Springer, 1993). Many developing countries, where the richest biodiversity occurs, lack adequate resources to develop its commercial potential and, in the absence of additional mechanisms, cannot expect to derive any economic benefit except over long periods of time. Clearly the development of a drug from natural sources consists of a research phase and a commercial phase (Table 2) and profits that can be shared are developed only during this later phase. For this reason, recent models for biodiversity prospecting have provided mechanisms to insure proper protection of both intellectual property rights and sovereign rights and developed just methods of compensation and profit sharing. These models have also provided a broader and more equitable view of the entire collaborative research process. Innovative collaborative research programs now being developed should combine the strengths of research groups in developed countries with those in developing countries. These should unite biology with pharmacology or biotechnology research organizations, and commercial with academic groups to provide benefits to participating developing countries during both the research and commercial stage of drug development.

Three recent models for drug discovery programs have addressed these issues. The first of these is the Letter of Intent, developed by the National Cancer Institute (Cragg et al., 1993). This document distinguishes intellectual property that can be considered both trade secrets and patentable inventions and it also addresses sovereign rights, including compensation and information transfer, and, in the broad sense of a collaborative program, addresses research opportunities and professional and

Table 2. Development of a Plant Derived Drug.

RESEARCH PHASE
Sample collection
Extraction
Screening
Isolation
Structure elucidation

Preclinical development
Clinical trials
COMMERCIAL PHASE
Licensing
Production
Marketing

institutional development. Certainly the entire NCI natural products program has provided innovative opportunities for collaborative research both in the field and in the laboratory.

Two additional models exist. One is based on the recommendations of Schweitzer et al. (1991), representing somewhat of an outgrowth and expansion of the Letter of Intent and a prelude to the International Collaborative Biodiversity Groups (ICBG) and the second is the Merck/INBio agreement. Like the Letter of Intent, both of these programs address all of the major issues outlined here, and their major difference is how they address the research period of drug development.

One major initiative of the ICBG's, a program jointly sponsored by United States National Institutes of Health, the National Science Foundation, and the United States Agency for International Development, is to enhance collaboration between research groups that normally only peripherally interact in both the developed and developing world. There also seems to be the clear initiative to address the problem that the biological resources most promising for natural products development are in the tropics while the financial resources remain in the developed world. There is a distinct recognition of the problem that drug development takes twelve years on the average and that innovative short and medium-term compensatory mechanisms are required to insure benefits to participating countries in the developing world.

The latter two issues, research opportunities and professional and institutional development, are among the methods available to provide appropriate benefit during the research stage of drug development. Clearly programs should be designed so that there is equal research participation among the collaborating institutions. Progressive programs will also provide components for training of staff and students and support for improvement of institutional capacity in developing countries.

A unique feature of the Merck/INBio agreement is that it has not only provided benefit to Costa Rica at the onset of the program, through direct compensation, but has developed the mechanism in a way that promotes the study and preservation of the

organisms of Costa Rica through programs that over time will increase the capacity of Costa Rican scientists and institutions.

These issues are not new and have already received a great deal of discussion. It is also clear that there is no single solution that will work in all situations. The Merck/INBio agreement may work well in Costa Rica and its basic philosophy might be applicable in other areas, but the precise mechanisms through which it functions may be neither directly transferable nor appropriate. As collaborative programs develop in the future, the most innovative will recognize that there are separate issues to address and remain flexible in adapting to local circumstances.

REFERENCES

Anonymous. 1993. WIPO. World Intellectual Property Organization. General Information. WIPO Publication No. 400 (E). Geneva, Switzerland.

Cragg, G. M., Boyd, M.R., Grever, M.R., Mays, T.D., Newman, D.J. and Schepartz, S.A. 1993. Natural product drug discovery and development at the National Cancer Institute. Policies for international collaboration and compensation, *in*: "Conservation of Plant Genes II: Utilization of Ancient and Modern DNA, " R.P. Adams, J.S. Miller, E.M. Golenberg and J.E. Adams, eds., Missouri Botanical Garden, St. Louis, MO.

Gollin, M. A. 1992. Carving property rights out of the public domain to conserve biodiversity. Paper presented at the Conference of the International Association for the Study of Common Property, Washington, D.C., September 18, 1992.

Gollin, M. A. 1993. An intellectual property rights framework for biodiversity prospecting, *in*: "Biodiversity Prospecting: Using genetic resources for sustainable development,"W.V. Reid, S.A. Laird, C. A. Meyer, R. Gamez, A. Sittenfeld, D. H. Janzen, M. A. Gollin, and C. Juma, eds., World Resources Institute.

Paddoch, C. and Boom, B. 1991. Draft Code of Ethics. Society for Economic Botany.

Paul, G. P. 1991. Project ABIGAILE: the story of the ABA and biodiversity. Diversity 7: 20-21.

Price, S. C. 1992. The economic impact of novel genes in plant biotechnology: not without strong intellectual property rights, *in*: "Conservation of Plant Genes," R. P. and J. E. Adams, eds., Academic Press, Inc., San Diego, CA.

Schweitzer, J., Handley, F.G., Edwards, J., Harris, W.F., Grever, M.R., Schepartz, S.A., Cragg, G., Snader, K. and Bhat, A. 1991. Commentary: summary of the workshop on drug development, biological diversity and economic growth. J. Nat. Cancer Inst. 83: 1294-1298.

Springer, N. 1993. R. & D. -- Spending anyway. Chemical Marketing Reporter March 8 1993:38-39.

BIORESOURCE-BASED OPPORTUNITIES FOR SCIENTIFIC AND INDUSTRIAL GROWTH IN DEVELOPING COUNTRIES

Kodzo Gbewonyo
BioResources International, Inc., P.O. Box 6595,
Somerset, New Jersey 08875, USA

SUMMARY

Bioresource-based opportunities are discussed in view of: trends in developing countries; traditional healers in Ghana; research in plant medicine; cottage pharmaceutical industries; export trade in medicinal plants; linkages of biodiversity and biotechnology; collaborative arrangements with developing countries; and screening for tropical diseases. From this a Bioresource Commercialization Strategy is formulated.

INTRODUCTION

During the past few years a number of economic, scientific and societal trends have culminated to refocus attention on the search for new drugs from the biodiversity of rainforests and other tropical environments. Primarily, the pharmaceutical industry faces increasing challenges to discover innovative drugs to tackle dreadful diseases such as cancer, AIDS and its opportunistic infections (drug-resistant tuberculosis and pneumonia).

In addition, mounting pressures on curtailing the high costs of prescription drugs have forced pharmaceutical companies to aggressively seek new drugs in order to expand market opportunities and maintain sales growth. Some pharmaceutical companies are experiencing dry spells in their drug development pipelines compelling renewed emphasis on discovery programs to generate new leads for development.

Besides economic motivations, the search for new drugs is fueled by spectacular strides in the biomedical sciences unraveling the biochemical mechanisms of major diseases such as Alzheimer's disease. These scientific developments raise prospects for new therapeutic targets for intervention in the disease processes. Application of recombinant DNA technologies has facilitated conversion of these new strategies into *in vitro* assays for drug discovery programs. The capability to mass produce target proteins - enzymes, receptors and reporter genes in whole cells has revolutionized *in vitro* screening of bioactive compounds for new leads in drug discovery (Edginton, 1992; Hodgson, 1992).

Automated techniques such as robotics have been incorporated in these *in vitro* screens to create a new generation of high throughput screens capable of handling thousands of compounds per day. These efficiencies in screening technologies shifted the limitations in drug discovery programs to the availability of chemical libraries for the screens. In this regard many companies are looking to biodiversity of tropical species as a virtually untapped repository of bioactive compounds to support drug discovery programs.

These revolutionary developments in screening technologies have spawned a proliferation of small entrepreneurial companies specializing in drug discovery programs against selected disease targets. The decentralization of the drug discovery program from major pharmaceutical companies represents an important structural change in the

organization of R&D in the industry. Since these new companies lack the internal stock of chemical entities to complement their screens, they are more willing to utilize plant extracts as potential sources of bioactive compounds for drug discovery.

Along with these scientific breakthroughs in drug discovery, a wave of public awareness has built up in favor of arresting the ecological threats to tropical rainforests and the potential loss of biodiversity. The conservation movement has drawn attention to the economic value of untapped plant species of the rainforests as potential sources of useful medicines which could be lost to mankind. An unusual alliance of interests has emerged between conservationists and the pharmaceutical industry regarding the intrinsic value of biodiversity in drug discovery (Jukofsky, 1991; Reid, 1992).

Finally the back-to-nature movement has also shifted consumer preferences in the west to natural products such as herbal remedies to common ailments. Herbal remedies are now sold openly in health food stores, although it is still difficult to gain FDA approval in the US for herbal remedies as therapeutic agents. This trend indicates a growing public acceptance into the mainstream of what has hitherto been regarded as alternative medicine.

TRENDS IN DEVELOPING COUNTRIES

As these trends gained hold in the developed countries, a different set of forces were at work in the developing countries which posses the bulk of the world's biodiversity. A sizable cadre of dedicated local scientists has developed during the last few decades pursuing research on the medicinal properties of plants used in local folklore. These scientists consist of ethnobotanists, natural product chemists, pharmacologists and clinicians investigating the efficacy, chemical characterization, distribution and taxonomy of medicinal plant species.

Some developing countries have initiated programs to develop medicinal plants derived from the local folklore as substitutes for highly priced imported medicines they could not afford to provide for their citizens. International health organizations such as WHO have now realized the predominant role of traditional medical practitioners in primary health care delivery in many developing countries. This recognition has led to measures to improve standards of practice and attempts to integrate traditional medical practitioners into the formal health care delivery systems in some countries (Akerele, 1984).

The renewed interest in the pharmaceutical industry towards the search for new drugs from plant sources has caught attention of scientists and policy-makers in the developing countries. In particular, the interest in ethnopharmacological leads for drug discovery vindicate the value of traditional medical know-how. The innovative legal agreements being proposed to compensate developing countries for access to biodiversity has generated enthusiasm and dispelled the aura of skepticism and suspicion that prevailed in developing countries regarding transfer of plant materials. This paper seeks to suggest that the convergence of interests created by these events offers opportunities for collaboration between developed and developing countries in efforts to employ biodiversity as a resource in the drug discovery process.

TRADITIONAL HEALERS IN GHANA

In order to provide a realistic context for discussion, a brief overview of the current status of medicinal plant use and research in Ghana is provided. The situation in Ghana is chosen because it is fairly representative of the West African region and also for

reasons of familiarity to the author. The significance of traditional use of medicinal plants in Ghana is demonstrated by the large number of traditional healers practicing in the country, estimated at 30,000 registered healers in a total population of 15 million people (Boye and Ampofo, 1990). Based on this estimate, the ratio of traditional healers to the population is 1:500 which compares to a ratio of 1:12,000 for Western-trained medical practitioners. More than 75% of the population, especially those living in the rural areas, have no access to modern health clinics and depend entirely on traditional healers for their primary health care.

These realities have led governments and international organizations, such as WHO, to recognize the influence and contributions of traditional healers and seek to integrate them in efforts to improve healthcare in developing countries. In Ghana an umbrella organization has been formed known as the 'Ghana Psychic and Traditional Healers Association'. This organization, recognized by the government as the representative body of traditional healers, certifies all qualified healers, establishes professional standards and codes of conduct, eliminates malpractice and excludes quack practitioners. A directorate for Herbal Medicine was also established recently within the Ministry of Health to coordinate efforts to integrate proven herbal remedies into primary health care programs.

RESEARCH IN PLANT MEDICINE

The government also sponsors a major research center dedicated to scientific investigation of Plant Medicine in one of the teaching hospitals in the country. The Center for Scientific Research into Plant Medicine was established in the early 1970s out of the efforts of Dr. Oku Ampofo, a Western-trained physician who did pioneering studies to validate the clinical efficacy of some traditional medicines. Dr. Ampofo's investigative methodology for working closely with the healers to document their practices (Hanlon, 1979) was incorporated in the scope of the Center's activities.

The Center consists of a Herbarium and Arboretum for cultivation and supply of selected medicinal plants under study and a Natural Products Chemistry Laboratories for isolation, purification and characterization of active constituents. Structure elucidation is carried on in collaboration with suitably-equipped overseas institutions. A well-equipped Animal Laboratory is used for pharmacological studies of plant drugs under investigation in small animals. The unique feature of the Center is the Outpatient Clinic manned by resident traditional medical practitioners alongside with Western-trained research clinicians who document traditional methods of diagnosis and prescription during consultations with the patient. The clinicians then conduct follow-up investigations to evaluate the efficacy of the prescribed herbal formulation in controlled clinical studies. The Center also runs a Pilot Production Unit for preparation of herbal extracts and formulations proven to be effective against common ailments, as well as a Pharmacy for dispensing herbal preparations to patients.

COTTAGE PHARMACEUTICAL INDUSTRY

With encouragement from the government, a number of cottage enterprises have developed within the past decade for small scale manufacture of herbal formulations. These commercial preparations are derived from generally accepted folklore as well as secret recipes developed by reputable traditional healers. Commercialization provides the advantage of standardized dosage forms of the preparations as opposed to the customized preparations dispensed by the healers directly to patients. Further, these

remedies provide inexpensive substitutes for imported medicines against common ailments such as toothache, piles and fevers. The preparations are properly packaged and sold in the informal retail sector through peddlers in local markets, bus and train stations.

The drawback to this burgeoning industry is that the preparations are not regulated, leaving room for abuse and adulteration often with fatal consequences. The preparations are claimed to cure a wide variety of often unrelated ailments, giving rise to skepticism about their true efficacy in some cases. Further there is little known about the chemistry of these preparations - the active ingredients have not been characterized. The toxicity profiles have also not been investigated and quality control in production is practically non-existent. No controlled studies have been done to demonstrate efficacy. Stability and shelf-life of the preparations have not been determined. The preparations are sold mainly by word-of-mouth recommendation from previous users. Since there is no patent protection, the formulations and the plant sources are held as trade secrets. In view of their vital role in society there is need to take steps to upgrade quality control, investigate efficacy claims and undertake chemical characterization of these products.

EXPORT TRADE IN MEDICINAL PLANTS

A thriving export trade in medicinal plants is developing in Ghana with approximately 100 tons of plant material exported annually, yielding modest revenues of 0.5 million dollars. The plants are generally collected from the wild since there is little cultivation of medicinal plants or sustainable harvesting practices. The supply chain consists of field collectors and a number of independent exporters. The plants are secured to medicinal plant buyers in Europe for distribution to processors who extract active ingredients for sale to pharmaceutical houses and other end-users. The main limitation on the growth of the export trade in medicinal plants is the poor flow of information about demand patterns from international markets to the suppliers in Ghana. This uncertainty in predicting demand makes it difficult to plan or engage in long term investment projects such as cultivation. The demand patterns are generally irregular due to competition from alternate sources of supply and displacement by synthetic substitutes.

The major plants currently secured from Ghana for international markets include:

i) *Griffonia simplicifolia*, which provides a plant lectin used in research and diagnostic tests for certain tumors (Lamb et al., 1983).

ii) *Voacanga africana,* which contains the alkaloids voacamine and vinburnine used in some hypotensive drugs (Newcombe and Patel, 1969).

iii) *Thamatococcus daniellii*, contains the protein sweetener thaumatin used as a low-calorie sugar substitute and flavor enhancer (Higginbotham, 1979).

From all indications the medicinal plant sector is primed for growth under the non-traditional export development program proposed by the Ghana Export Promotion Council (Odamtten et al., 1989).

LINKAGES OF BIODIVERSITY AND BIOTECHNOLOGY

The central paradigm proposed in this paper is that biodiversity is intimately bound to the biotechnology revolution and that the two phenomena could catalyze the growth of each other. This inter-relationship is illustrated in Figure 1. As noted advances in biotechnology provided the impetus to launch mass screening programs for bioactive products from plant sources. Similarly, the gene pools of biodiversity provide

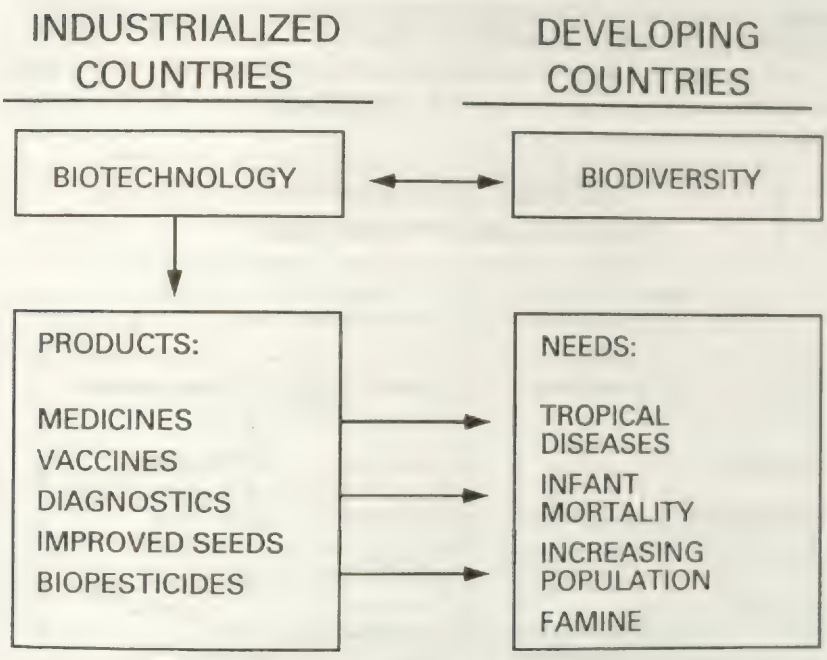


Figure 1: Linkages between biodiversity and biotechnology - Basis for collaboration between developing countries and developed countries.

templates for genetic engineering of new products (e.g., seeds) and processes. This synergy between biological resources and technology could be fully exploited by the parties controlling these factors of production in mutually beneficial ways (Taylhardat and Zilinskas, 1992). Developing countries face chronic problems which are amenable to biotechnological solutions. Problems such as endemic tropical diseases, infant mortality, low agricultural productivity in the face of rising population pressures can be addressed by biotechnology products, namely, effective drugs, vaccines, diagnostic kits, biopesticides, high-yielding, disease and drought-resistant seeds. Access to these products and the enabling technology are controlled by companies and institutions in developed countries desiring access to the rich biodiversity of developing countries. Provided the appropriate legal mechanisms and business arrangements, developing countries are in a strong position to leverage biodiversity for collaborative R&D programs in biotechnology and drug discovery with companies and institutions in developed countries.

An outline of the operational structure of a typical bioresource-based drug discovery and development program is shown in Figure 2. The operations consists of:

i) Field operations comprising plant collections (random and ethnobotanical), taxonomic classification, herbarium cataloguing and computer databases.

ii) Screening operations comprising plant extraction, *in vitro* assays of crude extracts in primary and secondary screens, purification of extracts, isolation and identification of active agent, and chemical structure determination.

iii) Development phase support activities include bulk collection, cultivation, pilot scale extraction and purification of active ingredients, drug supply for preclinical studies (safety assessment) and human clinical trials.

iv) Commercial production upon successful drug approval could involve large scale cultivation, extraction and purification to supply drug substance for formulation into final product.

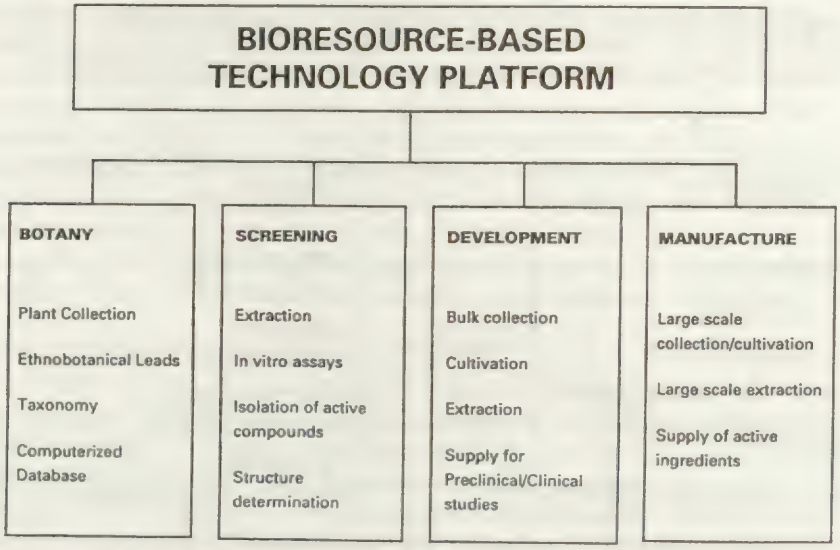


Figure 2: Overview of bioresource-based drug discovery and development program.

COLLABORATIVE ARRANGEMENTS WITH DEVELOPING COUNTRIES

Depending on the stage of development, infrastructure and human resource availability, developing countries may seek to participate in various activities within this technology framework contributing to scientific, industrial and economic growth of the country. Different levels of participation in the drug discovery program can be envisioned (Figure 3):

i) Primary level participation in the plant collection and taxonomy program in which plant samples are exported overseas to collaborating institutions. At this level of collaboration developing countries can seek support to strengthen botany programs, upgrade herbaria and botanical gardens, computer database development, inventory of biodiversity and conservation programs. The program sponsored by the National Cancer Institute to collect plant samples from tropical countries for screening against cancer and AIDS appears to follow this approach.

ii) Intermediate level of participation will involve extraction of plant samples to crude extracts for export and subsequent screening overseas. This stage will involve natural product chemists in the program with consequent strengthening of chemistry programs. One advantage to the developed country partner is reduction in freight costs as shipping bulk plant materials is minimized. Also repetitive, labor-intensive tasks(grinding, extraction) are relocated to developing countries reducing labor costs. One possible offshoot at this stage of the operation is to retain fractions of the crude extract in the developing country for screening against tropical disease targets. The collaborative program announced recently by Costa Rica (INBio) to supply extracts of

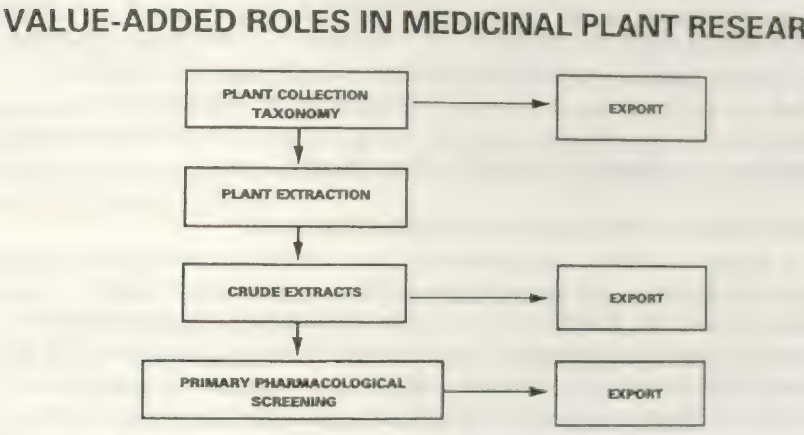


Figure 3: Models for developing country participation and collaboration in bioresource-based drug discovery programs.

biological materials to Merck for drug screening follows this model of operation (Joyce, 1991).

iii) Higher levels of participation in primary screening programs provide additional responsibilities to scientists in developing countries to conduct *in vitro* screens and select only extracts showing the bioactivity of interest for export. Further participation can extend to purification and structure identification of the bioactive compound. Participation at this level brings benefits of inclusion in patents as well as more comprehensive experience in the drug discovery effort. An example of this type of collaboration is the agreement sponsored by Syntex (Palo Alto, CA) to screen extracts from Chinese medicinal plants for bioactive compounds at the Hong Kong Institute of Biotechnology (Gwynne, 1992). A similar agreement was recently reached by Glaxo to set up a Natural Products Research Institute in Singapore for drug discovery.

SCREENING FOR TROPICAL DISEASES

Screening for bioactive compounds against tropical disease targets offers unique opportunities for scientists in developing countries to gain experience in the drug discovery process. Tropical diseases endemic to developing countries have been largely neglected in drug discovery programs by the pharmaceutical industry due to low economic incentives for these targets. However, considerable progress has been made in recent years in basic research studies of these parasitic diseases covering biochemistry, immunology and molecular biology. This effort was sponsored by the Tropical Diseases Research Program of WHO, which targets six tropical diseases, namely, malaria, trypanosomiasis, onchocerciasis, leprosy, schistosomiasis and leishmaniasis. The basic knowledge accumulated so far on the biochemical mechanisms of these diseases needs to

be translated into *in vitro* assay systems for drug discovery screens. Armed with these assays, developing country scientists can undertake extensive screening of plant extracts to identify bioactive lead compounds for drug development. These programs can be pursued independently or as part of the collaborations outlined above. Due to the magnitude of human suffering and economic losses caused by tropical diseases, developing countries must launch aggressive drug discovery efforts taking advantage of biodiversity to find effective compounds for these diseases.

BIORESOURCE COMMERCIALIZATION STRATEGY

It is clear from the discussion above that very promising opportunities exist at the interface of biodiversity and biotechnology for collaboration between research institutions, universities, private sector companies in developing countries and their counterparts in developed countries. One example of this role is demonstrated by the activities of BioResources International Inc. (BRI), the company with which the author is affiliated. The business strategy of BRI is to commercialize high value bioactive products from tropical plants for applications in the food, pharmaceuticals, cosmetics and specialty chemicals industry (Figure 4). BRI's field operations are based in Ghana

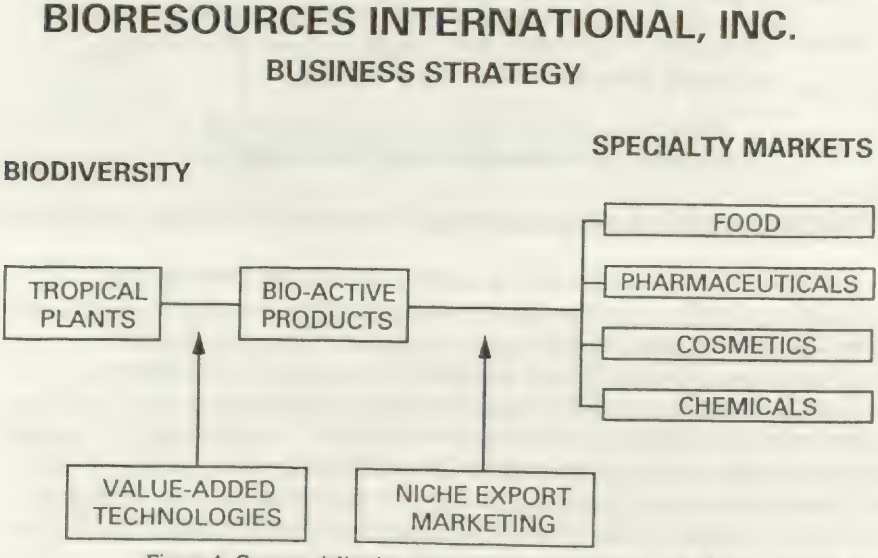


Figure 4: Commercialization of bioactive products from tropical plants - Business strategy of BioResources International, Inc. (BRI),

with a network of collaborations with biotechnology companies and university researchers in the US and Japan. BRI has targeted a class of high intensity protein sweeteners derived from tropical berries indigenous to West Africa for commercial development as food ingredients. Specifically, these products include Monellin from *Dioscorephyllum cumminsii*, Miraculin (taste-modifying agent) from *Richardella dulcificum* and Thaumatin from *Thaumatococcus daniellii*. These products have fascinated researchers and the food industry for sometime because of their extraordinary sweetness potency, however their availability and supply has hitherto been limited. Through the efforts of BRI the purified sweet proteins are now readily available at reasonable prices for research and development applications in the US. In Japan these

sweet proteins have received regulatory approval for use in food as natural products, consequently BRI is developing relationships with Japanese companies to bring these products to market.

In conclusion, the linkages between biodiversity and biotechnology have opened opportunities for scientific and industrial collaboration between developing countries and developed countries. It is anticipated that the evolving legal mechanisms will make these collaborations mutually beneficial to parties in the developing countries as well their developed country partners.

REFERENCES

Akerele, O. 1984. WHO's traditional medicine programme: progress and perspectives. WHO Chronicle 38(2):76.

Boye, G. and Ampofo, O. 1990. Role of plants and traditional medicine in primary care in Ghana, *in* "Economic and Medicinal Plant Research, Vol. 4," H. Wagner and N.R. Farnsworth, eds., Academic Press Ltd, London.

Edginton, S. 1992. Superfamily structure and biotech drug development. Bio/Technology 10:1529.

Gwynne, P. 1992. The Chinese biotech connection. Technology Review July p. 19.

Hanlon, J. 1979. When the scientist meets the medicine man. Nature 279:284.

Hodgson, J. 1992. Receptor screening and the search for new pharmaceuticals. Bio/Technology 10:973.

Higginbotham, J.D. 1979. Protein sweeteners, *in:* "Developments in Sweeteners, Vol. 1," C.A.M. Hough, K.J. Parker and A.J. Vlitos, eds., Applied Science Publishers Ltd, London.

Joyce, C. 1991. Prospectors for tropical medicines. New Scientist October 19, p. 36.

Jukofsky, D. 1991. Medicinal plant research leads scientists to rain forests. Drug Topics April 22, p. 26.

Lamb, J.E., Shibata, S. and Goldstein, I.J. 1983. Purification and characterization of *Griffonia simplicifolia* leaf lectins. Plant Physiology 71:879.

Newcombe, F. and Patel, B.A. 1969. Alkaloids of the leaves of *Voacanga schweinfurthii* Stapf. Planta Medica 17(3):276.

Odamtten, G.T., Laing, E. and Abbiw, D.K. 1989. Survey of Medicinal Plants of Export Potential from Ghana. International Trade Center, UNCTAD/GATT, Geneva. (Project No. GHA/50/08).

Reid, W.V. 1992. Conserving life's diversity. Can the extinction crisis be stopped? Environmental Science and Technology 26(6):1090.

Taylhardat, A.K. and Zilinskas, R.H. 1992. AGENDA 21: Biotechnology at the United Nations Conference on Environment and Development. Bio/Technology 10:402.

PANEL DISCUSSION ON INTELLECTUAL PROPERTY RIGHTS AND GENE UTILIZATION

Following the presentation of papers in the symposium on Intellectual Property Rights and Gene Utilization a panel of the speakers was convened and an open discussion was held on this topic. The following printed discussion was transcribed from a recording of the session. A special thanks is extended to Eloise Cannady and Nan Winkelmeyer who transcribed the tape.

Moderator:
Robert P. Adams, Baylor University, Texas

Panel Members:
Gordon M. Cragg, National Cancer Institute (US)
Kodzo Gbewonyo, Bioresources International, New Jersey
James S. Miller, Missouri Botanical Garden, St. Louis
Steven Price, Iowa State University

Participants:
Daniel K. Abbiw, University of Ghana, Ghana; Ravi Chibbar, Plant Biotechnology Institute, NRC, Canada; Paul Foster, University of Missouri, St. Louis; Sandra Knapp, National History Museum, London; Thomas McKnight, Texas A. & M. University; Irene Shonle, Washington University, St. Louis; James Zarucchi, Missouri Botanical Garden, St. Louis

Panel Discussion

McKnight: I have a question for Dr. Price. Most of the research at public institutions like Iowa State and Texas A. & M., where I am from, is funded by public moneys, either from the National Institutes of Health or from state sources, so I would think that the general public has some ownership rights to whatever comes out of that research. But when the patents are issued, they are all issued to the research corporations or to universities, or to the inventors themselves. How and why is that?

Price: That is a good question and one the public has addressed. Actually, over the last 12 years, approximately 10 laws have been passed, including one executive order, all of which address the issue of how to handle the disposition of intellectual property that comes out of publicly funded work. And what all of these laws say is that even though the work was funded by the public, it is okay to get patents on that work and it is okay for the contractor who is the recipient of the money, like Iowa State University, to hold title to patents. It is okay to give exclusive licenses. It is okay to keep the information confidential for what I find to be an unbelievable amount of time, five years. And it is okay for royalties to be generated and for the royalties to flow back to the institution or to the inventor.

So I would maintain that the public has indeed spoken quite clearly on this topic and the public is entirely supportive of a patenting, licensing, royalty-generation system. I would be more than glad to supply you with the titles of those laws and you can look

them up yourself. I find that actually many people are not familiar with those laws and, interestingly enough, I find that administrations, primarily in agricultural colleges, are unfamiliar with those laws, but their counterparts in engineering at the same universities have been functioning under those laws for the last ten years.

Adams: What restriction is there on the use of royalty? Is there a concept that the royalties that come from these rights that have been negotiated go back into a public institution, therefore the people are not really losing their money?

Price: I would be more Machiavellian than that. What the public recognizes is that inventorship requires incentive, and there is no better incentive than for an inventor to gain personally. This is a concept that goes back hundreds of years. Thomas Jefferson, who was involved in writing the first patent act, said that inventors shall receive a lucrative stimulus, and Abraham Lincoln was supportive of the same notion, so I think it is appreciated that in order to motivate faculty, and believe me, it is a very arduous process to make a dollar on technology, one of the compensations and incentives would be that the individual gets a share of the profit. I think that is the fundamental reason why these laws say very explicitly that the inventors shall benefit personally, because it recognizes the fact that in order to make it work, they need to have that incentive built into it.

Cragg: Hasn't there been a congressional investigation of the link between Scripps and Sandoz, the Swiss company, and a feeling of concern about having this link where Scripps gets perhaps, millions of dollars from the taxpayer, and all this expertise and knowledge will be passed on to one company.

Price: Yes. There the issue is not that the information is passing on to one company, nor is it exclusive licensing, but rather that Sandoz is a foreign company. There is a considerable amount of examination going on and those of us at universities expect to be put increasingly under the microscope for the kinds of deals we are doing. Recognizing that legislators are beginning to question relationships with companies and if they are Japanese companies or Swiss companies, what can be done to transfer this technology to benefit of the U.S. companies? That is the controversy.

Cragg: So maybe what is required is the normal government process of open competition.

Price: I can go on and on about this. In fact I would like to talk about open competition. The reason why many universities, ourselves included, are licensing to foreign companies is because U.S. corporations are sometimes not as competitive as many foreign companies. In fact I have got a very good case history. We made an invention in an anti-wrinkling, anti-acne compound, and threw it out for the world to bid on. No U.S. companies expressed interest in it. Two Japanese companies did, so we did deals with Japanese companies. Now, is that immoral? It turns out that deal rescued a laboratory that was without funding. So it is an example of how this process actually saved a research lab, which has since gone forward and gotten more NIH funding. So I feel very clean about that. The U.S. companies had a chance, but did not compete.

Miller: Using the Scripps/Sandoz case as an example, is the concern that Sandoz, rather than an American company, is receiving information from an American research institute, or that public funds are being used to generate results that are then being transferred out of the country?

Price: The latter. That is the concern and I think it is a legitimate concern. It is legitimate to ask the question, is the American taxpayer paying for research which then is going to a foreign company. It is a very good question, and we should be asking it.

Gbewonyo: Excuse me. As you negotiate these deals, isn't there an opportunity to levy a charge on basic research funding, especially where a foreign company is concerned?

Price: I like to have about five terms in every license agreement that I negotiate. One, I ask the companies to pay for the patent costs. Two, I like to get up-front license fees and third on the list would be royalties. Fourth, would be an exclusive arrangement. And fifth, would be that the company agrees to support research and that can be a substantial amount of money. Every year I generate about $400,000 in research funding that goes back to various laboratories as a result of this licensing activity. So for an institution like Iowa State that has a $120 million research budget, $400,000 is not a lot. But for some specific research programs it is a lot of money. And companies are willing to pay for that. It is cheaper for a company to pay for research in a university laboratory than it is to do it in-house, by a factor of four to five.

Adams: Steve, are there differences between states as to how public money is spent?

Price: It is not a state by state issue in general, although there are definite state differences in cultural attitudes. In their plant intellectual property, Nebraska is probably the most opposed to the issue of licensing plant materials for royalty generation. In Texas, and I know my peers from Texas A. & M. rather well, they are very supportive of licensing plant materials for generation of royalty. It is controversial on campus, but the institution is going ahead and doing it anyway. I find that, rather than controversies being state by state, usually the most vocal opposition to licensing plant materials for royalty generation are found in colleges of agriculture. Since they have traditionally given out plant materials free of charge, the idea of generating royalties is pretty much the opposite of the way they have been behaving.

Chibbar: I would like to comment on the first answer that you gave. When you generate money for research, as in the five points you raised, does that money go for further refinement of that product or invention, or can the scientists who did the work use it for new ideas?

Price: It depends on the deal and the faculty member. Sometimes the faculty member does not have a continuing interest in the developed technology, but in general, I find that industry is most interested in supporting the further development of that technology. Most faculty are pretty adamant that they are not hired to do, nor do they have an intellectual interest in, developmental research. As long as we can keep the research in the applied area, most of the time that is acceptable to the faculty. Industry understands in general that they are dealing with a research culture and not a development one.

Foster: I have a question for Dr. Cragg. Referring to the recommendations of Dr. Gbewonyo, what do you think of his suggestion of preparing extracts in the country where the plants are collected rather than shipping the plants back to Frederick, Maryland. Is that feasible or is that in the long-term plans for the project?

Cragg: Yes, I like that idea in the long term. The immediate reaction of some colleagues is quality control, but that is something which I think can be handled. I certainly like the idea and I think the idea of value-added products being exported is something which countries should be promoting. It is just a matter of getting the necessary infrastructure in place to handle that and also assuring the recipients that the quality of the material is acceptable.

I wanted to add something. In his paper, Jim Miller mentioned the International Cooperative Biodiversity Groups which are being set up by joint sponsorship between the NIH, the National Science Foundation, and USAID, and this is a very exciting program, I think, because one limitation which we have had at the NCI is that we feel we cannot put money into infrastructure development. We just have not got that sort of funding, whereas USAID, of course, can support that very strongly. So this would be a mechanism if laboratory facilities need to be improved, or standards of herbaria, etc., and that sort of money could be channeled through USAID, where NCI would not be in a position to provide funding for that sort of development.

Gbewonyo: I just want to add that with regard to fears about quality control, I think the scientific procedures will take care of that. You can always incorporate internal standards and monitor that the job is being done right and it should not be such a big problem.

Cragg: No, I agree. I think it is clearly manageable. We have definite protocols at the NCI for the way we extract, and those can be transferred very simply in fact. So I think it is a perfectly feasible process.

Miller: I think it is necessary for people to realize that in-country extraction is a difficult thing to have happen overnight. Regardless of what your extraction methodology is, it has to be consistent. The other thing is that innovative programs are going to realize that, as Kodzo implied in his paper, it is cost effective to bring a lab in a developing country up to a certain level, enabling them to perform consistent extractions. These costs may be offset by reductions in shipping costs and the high price of laboratory space in the United States. So a lot of these value-added factors are to everyone's benefit, although a greater part of the total costs may be moved to initial stages of a program.

Knapp: I just wanted to mention that there are several programs going on with European pharmaceutical companies who are proposing to do exactly the same sort of thing: to set up labs in developing countries in collaboration with the governments of those countries. In this way the governments of European countries can be seen to be complying with these requirements, and having signed the biodiversity convention are required to do things just like this.

Miller: Are these European pharmaceutical companies, private concerns, or both?

Knapp: The companies I have been dealing with personally are pharmaceutical companies and they have an interest in establishing the laboratories in developing countries to ship out extracts. In any negotiations into which we have entered, which have only been tentative at this stage, we have been very clear that the benefits of this should definitely go to the developing country and not to the intermediate provider.

Cragg: I do not think that many U.S. companies would have any problem with that because the extraction technology is not proprietary type information. It is very standardized and publicly known. I think that the major problem U.S. companies had was with proprietary information. I would say this was particularly true in the biotechnology area, the subject of Rob Horsch's paper, but again, these are problems which can be handled.

Knapp: This will come more to the forefront as more and more countries become signatories to the Rio Convention, which will just require us all to address these problems directly.

Abbiw: Dr. Cragg, another issue that came up when your colleagues Jim Miller and Pete Lowry visited Ghana, was that the NCI looks only at AIDS and cancer. We in Ghana are plagued by many more diseases than these and would like to see other ailments receive attention as well.

Cragg: I, in fact, applaud that attitude. The mission of the Cancer Institute for which we are funded is cancer, primarily, and AIDS has been included in the last four or five years, and it is a very defined mission. We just would not be permitted to alter our mission statement to screen for parasitic diseases and so forth. However, through our Letter of Intent, we are very happy to have the in-country organization with which we are collaborating through the Missouri Botanical Garden screen those extracts for other activities. In fact, we would encourage that.

There is also a possibility through the Material Transfer Agreements, the latest mechanism we are developing to distribute extracts to other interested and very carefully selected companies for screening in systems which reflect diseases of importance to the countries where the plants came from. My hope is that we will get other organizations to express interest in screening for diseases of importance in the source countries. I certainly would encourage those of you working through the Missouri Botanical Garden to have extracts which you keep and screen for activities or relevance to your particular needs.

Miller: One of the things that I mentioned this morning was the chance to increase research opportunities through these programs and bring potential benefits into an earlier phase of drug development. We can come up with modifications like collecting slightly larger samples so that a portion can be left to be studied in-country with the primary focus of health issues that are locally relevant rather than those most important in the developed world.

Gbewonyo: One question raised by Dr. Cragg's suggestion to screen in other areas than cancer and AIDS is how does the NCI's Letter of Intent control transfer of material to a third party?

Cragg: We now have a separate document, called a Material Transfer Agreement, and within the terms of that are clauses which state that the recipient of those materials must abide by exactly the same terms as stated in the Letter of Intent. The most relevant terms are royalty payments or some form of compensation should they discover a good drug and also use of the source country resources for further development and eventual commercial production. That agreement does cover the fact that they must abide by the same terms as the Letter of Intent.

Knapp: Who polices the thing?

Cragg: We would. The National Cancer Institute would. We have also been questioned about the phrase, which appears in our Letter of Intent, "make best efforts" and I thought I should elaborate here that as a government agency by U.S. law we cannot state we guarantee that this will happen, but this Letter of Intent has the enthusiastic and very strong commitment up to the highest levels of NCI, NIH, and the Department of Health and Human Services. I am hopeful that support runs to the highest levels of the administration.

Price: I am sitting here thinking about the consequences of Intellectual Property Rights as the extraction and screening take place in the source countries. This means that there will be an increasing chance of the source countries coming up with the patentable discovery, which is defined as something that is unique, useful, and not obviously known to the world. With the current movement in patent harmonization that is certainly going to be adopted by the world in the next couple of years, it will mean that when you file in a source country, it will set a priority date with the rest of the world. I wonder if you are going to find that companies are going to be asking for changes in the language to make sure that they in fact receive all their rights. I predict that will happen.

Adams: I think that is a point that we need to be aware of in negotiating things. As we turn out a more screenable product, we have increased its value and need to be asking for a little different kind of royalty reservation. That has to be very clear and negotiated of course, but I think that is going to be very useful in the future.

Cragg: Yes. This is part of the value-added incentive. In my paper I alluded to so-called random as opposed to ethnobotanical or ethnopharmacological selection of a plant. If a plant is selected based on ethnomedical observations and that information is presented, then I think that grants the provider of the plant co-inventorship. But a random collection would not be, and Steve can correct me, regarded as granting the collector of the organism some co-inventorship.

Price: That is true, but if the collector or whoever in the laboratory actually recognizes the invention and finds it is active against AIDS is lucky enough to determine what the active component is, they have it.

Gbewonyo: I just wanted to get a clarification from Dr. Cragg. Are you saying that if a plant is used medicinally for disease X in a country and is found to be active against cancer, that entitles you to some intellectual property rights, or that it has to be used ethnopharmacologically against cancer before you get entitlement?

Cragg: That is an interesting question. Personally, I think that if the plant was selected on the basis of some ethnomedical use and if it could be remotely connected to cancer or a viral disease in the case of AIDS, I think that would be a justification for co-inventorship. I am not sure whether the patent office is going to agree but Steve can comment on that.

Price: It is not up to the patent office. It is really up to the attorneys that draft the patent application, so if you were to hold firm and to insist on joint inventorship and therefore joint ownership, which follows logically in my opinion, then you would be in a strong position. I would just hope that you would hold out for those possibilities in whatever agreements you are negotiating.

Miller: There are a number of people, Paul Cox and Michael Balick, for example, who have argued that any ethnobotanical usage, be it related to the therapeutic area that you are screening in or not, is in effect a prescreen for general bioactivity and hence increases the likelihood of yielding activity.

Cragg: I agree entirely with that. Any ethnomedical use indicates that there is something bioactive present in that plant and therefore you have had a very distinct contribution to the selection of that plant.

Zarruchi: When you mention random searching, if you target members of a certain plant family like Apocynaceae or something else known to be chemically interesting, is that a random search or targeted and therefore subject to joint co-inventorship?

Cragg: Actually, some of our collectors have suggested that they should focus on certain families because those families have a history of producing interesting bioactive molecules. We tend to discourage that in fact because as far as cancer and AIDS are concerned you just never know what you are going to come up with next. The *Ancistrocladus* with Michellamine was a random collection. Duncan Thomas just happened to find this vine growing on a fallen tree in the Korup region, and decided to collect it. Even the *Taxus* that is the source of taxol, *Taxus brevifolia*, was collected by a USDA botanist on his way out of the Pacific Northwest. He just saw this rather undistinguished-looking tree and thought he might collect some of it. So that is why we encourage this broad taxonomic diversity, rather than say that this family is known to produce interesting alkaloids, therefore we will focus on that family.

Zarucchi: I have a further question in reference to targeting certain groups. When I collected for the NCI in Madagascar, we were collecting randomly, but were asked to make a special effort to collect taxa endemic to Madagascar. Given the high level of endemism in Madagascar and the fact that this was the first collecting done for the NCI in that country, it was a means to find plants that had not previously been studied. Is that targeted?

Miller: There are a number of ways to design sample gathering strategies. Clearly there is intellectual input in an ethnobotanically guided program. There are also taxonomically guided procedures. For example, you might want to look at the other species of *Taxus* for taxol analogues or if you presuppose that you are interested in benzylisoquinoline alkaloids, you only want to look at the few families in which they occur. Clearly, there is also intellectual input in these programs as well.

I would propose that so-called random collecting should be a random sample of taxonomic diversity and not merely unguided collecting. This is what Jim is alluding to. When he and Amy Pool collected in Madagascar, the focus was on families and genera that were endemic and had not been previously screened. The philosophy was that by actively searching out these taxa, it would be likely that we would increase the cumulative chemical diversity going into the screens. Whether or not the intellectual input in this is comparable to the ethnobotanical or taxonomic approaches, there is definitely a certain amount of botanical knowledge required and I would propose that this constitutes a significant intellectual input.

Price: I have been listening to the questions relative to randomness and I interpret the question slightly differently from the others. The issue of random versus directed for the purpose of defining inventorship is a very important area and it is also a gray area. There is a legal definition of an inventor and it is different than the definition for scientific authorship on a paper. The definition is narrower, but the acid test in asking if an individual is a joint inventor is whether the person had an intellectual input into the discovery. If a person selects a certain genus or family based on ethnobotanical information that the plant has activity against a disease and selects the plant for that reason and that is documented, and then it is found by someone else that extracts from that plant indeed do have activity, I would argue that the first person is a co-inventor. He is not a sole inventor, but a co-inventor.

Zarucchi: So you are saying that the botanist out in the field making the selection is the co-inventor.

Price: If that person is making more than just a random collection, but if he or she consciously selects the plant through an educated guess or other information, and the active properties are borne out, I would argue that is an intellectual input and that person should be a co-inventor.

Zarucchi: Okay.. The intellectual input that I am thinking about is not just chemical information, but based on the classification of the plant.

Price: So you are saying that plants in this family...

Zarucchi: That there is intellectual input necessary to identify them there in the field and know what you are collecting.

Price: It is an interesting point for discussion. It is a discussion that attorneys would have to sit in on and ultimately would be a legal decision. If you can make the point that you had an intellectual input and without that intellectual input the discovery would not have been made, then I think you are on very good grounds. At Iowa State University, you would be on very firm grounds for being a co-inventor.

Miller: I have a question for you Steve. I think we are arguing that there is intellectual input in almost any sample gathering that a botanist does; that we are actively thinking about all of the things we do. Obviously we are not capable of walking out and picking up one plant that is the cure for a disease, so how does that fact that hit rates are very low, perhaps one in 10,000 or one in 50,000, affect the idea of botanists being co-inventors?

Price: If you are consciously focusing on a genus or a family because of some knowledge that you have that would lead you to believe that members of this genus would have bioactive compounds, then that would do it.

Miller: There is a certain degree of unpredictability here. I can collect 99 plants for a very specific reason and the 100th simply because I am there and have adequate time. Hence there may have been enormous intellectual input into the entire program, but what if the 100th plant is the successful one? Can the intellectual input be in the design of the overall collecting strategy, or do you have to build a case for each individual sample?

Price: I think you should have it documented in a field notebook. This is not a trivial point. In most industrial laboratories, you will find that they are very strict about how they keep their lab notebooks as to when they had ideas and what they did. The reason for that is so they can demonstrate when they had the idea for purposes of intellectual property protection.

Knapp: I think this whole discussion of legal rights is very interesting and it is an area that is very gray and needs a lot of discussion. For example, if you are in the Amazon rainforest and you collect a plant which is known against viral diseases and you collect it in one tribe in one area, but if this plant is used throughout the Amazon by all tribes in many different countries, where do the property rights go in that particular case; to the shaman, to the tribe, or to the country? These are all very interesting legal questions that have not been addressed.

Price: It is an interesting question, but I would say the example of use throughout the entire Amazon may in fact prohibit patenting because the patent law says that you cannot obtain a patent on something that the public knows about. If it is generally known that a given plant cures a certain ailment, this fact could perhaps be used to claim that it is not patentable.

Knapp: This is why people do not investigate dysentery cures, or cures for common diseases in third world countries, because plants that are used against those are common knowledge. Is that not true?

Gbewonyo: I think that a lot of the very good herbalists protect their know-how. They do not have patents, but they have trade secrets which they protect and then it is not common knowledge but rather knowledge that is protected and handed down within a family or within a small community.

Knapp: In my experience in South America there are many, many examples of plants that are used widely throughout the region.

Adams: You can go to a marketplace and find certain plants that are for sale and everybody knows you take these when you have a stomach ache.

Miller: But it is the plant that is widely used and the plant is not patentable anyway. However, the purified biochemical that is responsible for the observed activity would still be patentable because there is further intellectual input in isolating and characterizing the compound. It is not an obvious extension of public domain information.

Price: Right. It is the same argument that I used for getting you to be an inventor to begin with. Maybe you brought some intellectual knowledge that was not generally known to the world that got you to the point of being a joint inventor.

Knapp: The patentable bit in something like that would be the extraction, or the compound itself, as opposed to the plant that it came from.

Cragg: I think that this sort of instance has arisen with Shaman Pharmaceuticals. I think that the plant from which they have isolated their promising agent is widely used throughout a number of countries in South America. They have what they call the Healing Forest Conservancy. It is an organization which is going to disperse benefits to a whole range of different countries and organizations and it might be that it was planned this way because so many of the plants that they do collect are used through a wide range of countries. The terms of our Letter of Intent exclude plants that are common weeds or crops unless that particular plant has a use in the country of origin. We feel that even if a plant is a ubiquitous weed, there is a distinct contribution to its selection if it is used in a given country for treatment of a particular disease.

Knapp: A lot of uses of widespread weeds could be convergent discoveries, rather than uses that spread with the weed.

Gbewonyo: Isn't that a case in which the first-to-file principle is relevant?

Price: In that situation if the discovery is in country A and country A files first, they would have priority.

Adams: Let me ask this. If you have country A and they can document that they have been using the plant as a medicinal for 1,000 years and country B, which has been using it for 200 years, then you might have another interesting problem.

Price: I think there is probably no case law on this.

Shonle: This is sort of another derivative of the co-inventorship question. What do you do when you are working with a particular group of people and it is common knowledge among that group of people, but not among widespread groups?

Adams: Well actually Steve could answer that question if you consider the parallel basis of a lab. If you are working in a research lab and everybody knows what you are working on and you talk about it, there can be problems.

Price: Well, it is kind of a gray area. We do have analogies to the situation that Bob just described when there are people working in a large lab and the results are generally known to everyone in the lab. The question is whether a sample of 15 people, if that is the size of your laboratory, is a broad enough sample for one to make the argument that it represents the public. If it is still known only within the confines of the lab, I would argue that the public still does not know.

Adams: Aren't all 15 of those people legally co-inventors?

Price: No. Those people are only co-inventors if they have had intellectual input into this.

Adams: Well if anybody had knowledge, wouldn't you have to include them on the patent with you?

Price: A patent can be invalidated if there is someone who is truly an inventor that is not listed. But that person has to be able to demonstrate that they had an intellectual input into the invention. Now if the invention arose during a laboratory discussion and everyone was involved in a free interchange of ideas and out pops some invention and you are unable to identify who had the idea, then I think an argument could be made that all 15 people would become inventors. The patent office does not care how many people are listed on a patent application.

Miller: Michael Gollin, a Washington, D.C.-based lawyer, has addressed these exact issues. In answer to Irene's original question, the real issue is if within a tribe their knowledge is subject to efforts to maintain its secrecy? If that is true, it should be subject to legal protection as a trade secret.

Cragg: But what if that information is disclosed to a collector, for example, an ethnobotanist?

Miller: I believe that if it is published, that puts it into the public domain, hence it would be tough to still consider it a trade secret.

Cragg: That is a very interesting point. We do have collections from certain tribal federations in South America and we are busy now negotiating Letter of Intent-type agreements with certain of these federations. I wonder if a representative of the federation, generally agreed upon by the people, could be included as a co-inventor?

Price: No. You have to get all of the inventors on the patent application.

Zarucchi: You brought up the question about when information has entered the public domain. Is information written on an herbarium label that is deposited in a public institution in the public domain?

Price: Yes. In fact, a thesis filed in the United States is considered in the public domain, even if no one looks at it. In the United States we would have one year from that date to

obtain a patent. In other countries of the world, you would be barred and could not obtain a patent.

Zarucchi: And this is retroactive?

Price: In the United States you have a grace period of one year from the time that you have enabled the public to file a patent application. After 366 days it is over.

Abbiw: In Ghana, some traditional healers have a plant that they use to cure snake bites. In order to continue to maintain the secret, it is administered in a mixture with about six other species of plants to hide the identity of the active one.

Adams: Well, that is definitely proprietary information and a good example of a trade secret.

Chibbar: Can anyone comment on the possible situation where a botanist discovers a plant that produces a particular active compound and then in a biotechnology lab, someone is able to enhance production by cloning the gene. Does the person who clones the gene get the patent or do both of them?

Price: I would argue that this discovery would not have been possible without the tribal healer's information indicating efficacy for snake bites. If this information leads to the discovery of the gene for a particular protein that is an antidote for snake bites and a patent application, you could argue that the traditional healer is a co-inventor. He is not the sole inventor, but a co-inventor and if I were negotiating for him, I would argue that he should benefit equally from any royalties that would be generated.

Knapp: You can also have the situation where a plant is used to treat snake bite because it is something that reverses anticoagulants in the snake bite, but the healer does not know the exact function of the plant. He knows it is good for snake bites. I have heard people from pharmaceutical companies say that we are looking for coagulants and since he did not know that was a coagulant, he does not deserve co-inventorship.

Price: That has not been the case at Iowa State and in fact attorneys looked at the situation and said that the individual is an inventor. I can give you the case that is analogous to this. One person said that a grain had utility for X purpose. I will not tell you what because that is proprietary, but it does not matter. It had X function. He handed it off to a second person who determined what the active ingredient was. The attorney believes that there is a very solid basis for both individuals being considered inventors. It seems to me that this situation is completely analogous.

Adams: I think she is talking about something a little different here. Can you repeat your question?

Knapp: Well, if you have a plant like *Brunfelsia*, which people say is useful against aching bones, a pharmaceutical company can isolate an active ingredient for a particular

biochemical purpose and they could say they were looking for an anti-inflammatory, not a remedy for aching bones, and therefore they do not share inventorship.

Price: I think that is weak logic.

Miller: I do, too.

Adams: I think the case that you gave originally about the snake bite cure is even more convoluted. The medical usage of a coagulant is completely different from a snake bite cure.

Miller: I would argue that the discovery would not have been made without both the knowledge that a given plant was used to treat snake bites and that this might be an indication that it had anti-coagulant properties. There is intellectual input from both parties.

Knapp: I would argue it that way, too. There are examples of fish poisons that are used as insecticides.

Price: There I think you are on weaker ground. In a hypothetical example, a plant used as a snake bite cure might be found to increase milk production, a totally unrelated and unpredicted function. Then I would argue that you are probably on weak ground in claiming that a healer would be a co-inventor.

Knapp: It would be on weaker ground the first time a snake bite cure that is an anti-coagulant is used to increase milk production. The next time another company might say we want to increase milk production and it has previously been done from a snake bite remedy, let's go out and find other snake bite remedies.

Price: Well, as I understand the example, the case law is rather clear. The second company probably would not be able to get a patent because it could be argued that the world already knows, from the issuance of the first patent, that anti-coagulants result in increased milk production and that would probably bar anyone else from obtaining a patent.

Gbewonyo: Over and above the patent mechanism, there are other legal mechanisms like the NCI agreement, which compensate for leads from botanical knowledge. In some respects, this is outside of patent issues. Compensation is possible without actually being named on the patent. Is that correct?

Cragg: I guess that is right. In the instance of the Letter of Intent, the organization or person who actually provided the material does not necessarily have to be a co-inventor on the patent to benefit eventually from royalty payments should the NCI develop a good drug from that plant. They do not have to be co-inventors.

Adams: It seems like there is a great take-home lesson in this discussion and that is to get agreements resolved up front about what compensation there is going to be, because patents may or may not come to fruition for a number of different reasons. I think that

we should be focusing a little more on licensing agreements and other agreements; documents that are just as important as patents.

Price: Just because you are a co-inventor does not automatically mean that you are going to share in any commercial activity or royalties. The terms of license agreements have to address not only inventorship, but also royalty sharing. The law is pretty clear in the United States, and I would argue that if there are trade secrets involved you can insist on royalty generation from those. In fact, that is more attractive than royalties generated from patents, because patents last only 17 years and trade secrets have no expiration, so you could enjoy royalties for much longer than 17 years.

Adams: I want to ask about the patent law. In the case of drugs, has that been changed to allow extension beyond 17 years?

Price: It is 17 years. What you are thinking of is that if a company can demonstrate that a patent was issued in year one, but it was not until year 15 that they were able to commercialize it because of regulatory delay, then there can be a case by case extension.

Adams: I believe that someone said that the average drug took 12 to 14 years to reach the market. If you take 12 to 14 years off of a 17-year patent, that would not give you much time.

Price: There are ways of legally delaying the issuance for a long period of time.

Adams: I know there has been discussion about changing that law to 17 years after a drug becomes commercial, but that has not been passed yet.

Price: I don't think that will ever be popular. I am unaware of that kind of discussion even in other parts of the world. What is being considered in these patent harmonization discussions is that a patent will be good for 20 years after the first public disclosure or 20 years from the time of filing a patent application.

Adams: So a lot of drug development must be done in secret for a number of years to avoid disclosure, which starts the clock ticking. I know this is a major concern.

Price: Trade secrets are to a company's advantage, absolutely.

McKnight: At what point does NCI think about starting the patenting process on these drugs?

Cragg: We will only take out a patent on an isolated pure chemical from the plant which demonstrates significant activity in our in-vitro screens. Generally, before applying for the patent there will be a certain amount of secondary testing, but there has got to be some demonstration of efficacy in the screen as well.

McKnight: So how many patents have resulted from the 65,000 extracts that you mentioned in your paper this morning?

Cragg: So far, I think the chemical isolation group have applied for four patents. These are pure compounds that have been isolated and demonstrate significant activity which is considered by the NCI to be sufficient to warrant preclinical development. Sometimes we get compounds isolated which show interesting activity but are not considered good prospects for preclinical development; therefore, we will not patent, we will just publish.

Adams: Are those compounds made available, or the knowledge of those compounds made available, to the public who might do some structure-related chemistry to change the activity?

Cragg: Those results would be published through the normal journals.

Adams: So we could pick up on this and get some of the material, go to our labs, start modifying it, and perhaps make something.

Cragg: And generally with compounds which we consider to have significant activity and are going into preclinical development, we hope to put out an announcement once or twice a year to encourage the research community to get involved, looking at synthetic modifications, total synthesis, or possible cultivation of the plant material and so forth.

Adams: Who owns the property rights for future screening of the extracts that you have screened for AIDS and cancer? Are your agreements written so that if ten years from now we have an epidemic of some bacterial infection and someone screens these extracts, are your agreements retroactive for just cancer and AIDS?

Cragg: Yes, the Letter of Intent will still apply. If an extract collected in Ghana has been in our repository for 20 years and 20 years hence suddenly shows remarkable activity against a new strain of tuberculosis, Ghana would still reap the benefits of the Letter of Intent.

Adams: I ask this because it is a question that comes up in DNA banking. You can use a gene that has been banked from a plant and put it into a bacterium and make a novel protein. I think that someday we will be able to sequence genes and probably tell something about what they do by their sequence. That is kind of a far-fetched idea right now, but I think the day is going to come when you will search a bank of sequences and look for interesting variants that have evolved in different areas. These will be put into an organism to see if you do get a different kind of activity. This is going to get very difficult, particularly where we are trying to store duplicate materials. One of the recurrent themes in DNA banking has been that we need to back up DNA banks; that we should not have only one copy in the world of something that is endangered. But that implies a tremendous amount of openness and sharing of those genes and the idea that has evolved is that we will PCR the products for duplicate DNA distribution. No original DNA would be given away, but only the PCR product.

The use of these duplicate materials raises a difficult legal question. It is a question that I have been asked several times and I wonder if the panel has any thoughts on this.

Price: I think the world has a lot of experience with this. To me it is completely analogous to the way companies handle requests for chemicals that they have for purposes that they do not envision. If you contact a company and say I would like to have compound X and I want to try this or that test, which may be completely outside the realm of the company's interest, they will send you a Material Transfer Agreement. Their agreement may say that they own the right to all patents that emerge from that material. That is their standard opening, but it may be negotiated to joint inventorship or ownership and most companies find that acceptable. These are not expiring Material Transfer Agreements and anything that comes out of their material, regardless of time, means that you are bound. I think the test of these is going to be what institutions are going to be around long enough to monitor them.

Adams: That's right. If you make an agreement with the government of Haiti, for example, I would say that right now it is pretty academic.

Price: Would you have asked the government of Haiti to sign an agreement or would you have asked the institution? The government of Haiti may come and go, but the institution is probably going to remain there.

Gbewonyo: I know that in most of biology, Material Transfer Agreements are routinely used, but I have been uncomfortable with how effective Material Transfer Agreements are with single plants exchanging hands.

Price: We use them all of the time at Iowa State University. There is not a seed that goes out of the institution, or even from professor to professor at the institution in some cases, without a Material Transfer Agreement.

Gbewonyo: While we talk in terms of massive collection programs like the NCI's, a lot of one-on-one single plant transfers which go on, particularly from developing countries, are literally unprotected and the use of Material Transfer Agreements are the key to protecting them.

Cragg: That is why I was very interested in your comment this morning that there is increasing emphasis on legal mechanisms being developed in Ghana. I think that is very important and would be welcome by most developed countries, too. It is a means of protection of intellectual property rights being legally developed within the developing countries. Of course, that would give a measure of confidence to many of the companies in the developed world that their intellectual property would be protected in the developing world as well. I would like to ask Steve to discuss the difference between inventorship and ownership.

Price: It is very simple. A condition of employment for most companies is that you sign all rights of ownership over to the company, but you can still be an inventor or co-inventor. The scientists are listed as inventors or co-inventors, but the company has ownership. At most universities, whenever researchers get any research money, they agree to sign all ownership and patents over to the university, but they are listed as inventors.

Cragg: Can the owner of a patent then assign part ownership to another organization?

Price: Sure. Patent ownership is like any asset.

Adams: Our time is over. Let me congratulate our panel and the participants for an enlightening discussion and the interesting dialogue on Patents, Intellectual Property Rights, Licensing Agreements, and Material Transfer Agreements. Thank you all.

COMMENTARY ON MISSOURI BOTANICAL GARDEN SYMPOSIUM DISCUSSION

Michael A. Gollin
Keck, Mahin & Cate
1201 New York Avenue, N.W., Penthouse
Washington, DC 20005-3919, USA

Several challenging issues weave throughout the panel discussion at the DNA Bank-Net symposium on tropical genetic diversity: (1) public policy towards technologies developed with public money; (2) mechanisms by which those who provide genetic resources and botanic information can share benefits derived from use of the samples and information; and (3) how to determine whether a traditional user of a plant or a collector is an inventor of a resulting drug or compound. These issues are considered below.

1. What rights does the public have over technologies developed with public money?

As noted in the discussion, several federal laws govern the allocation of rights in technology from government-funded research and development. In general, whoever pays the piper calls the tune, and the granting authority may set whatever standards or obligations are appropriate. One must keep in mind the different policy goals of the different grant programs. If we are simply seeking to fund a broad range of domestic research, intellectual property rights should be allocated to the innovators to help stimulate research and advance of technology. If we are looking to provide a subsidy or "economic stimulus" to particular industrial sectors, or to protect genetic diversity, different standards may apply.

In the context of genetic prospecting, the standard of sustainable development requires harmonizing the policy of promoting technological advance with the requirements of the Convention on Biological Diversity, which reflects the now famous land ethic of Aldo Leopold. "A thing is right when it tends to preserve the integrity, stability and beauty of the biotic community. It is wrong when it tends otherwise" (Leopold, 1966). That is, public funding of a program to sustainably use genetic material should be evaluated on the basis of the program's effectiveness in promoting local habitat conservation. This test will not necessarily be wholly consistent with the goals of promoting domestic industry and biotechnical research.

In genetic prospecting arrangements, the land ethic favors greater benefits for the person closest to or with the greatest control over a habitat, such as the person providing plant extracts, as an incentive for conservation. These benefits may include ownership of intellectual property rights, cash payments or royalties, participation in research and access to technology. Building a biotechnology infrastructure in source countries can also provide incentives to conserve genetic diversity, and can help address local health and agricultural needs.

The goals of promoting research and supporting domestic industry, in contrast, may be better served by providing greater benefits to a domestic pharmaceutical company that is screening the extracts for biological activity. Of course, in the long run,

destruction of biodiversity can be expected to impoverish everyone from suppliers to researchers, to the public at large.

An example of a policy consistent with the land ethic is NIH's program for funding International Cooperative Biodiversity Groups. NIH dictates that proceeds from commercializing biodiversity must flow back to the source country and be used for a conservation purpose. In this case, the public benefits indirectly from such targeted grant giving in ways that may be more important than maximizing the royalties that would flow back to the treasury.

Another example of a policy of local benefit is found in Article 9(a) of the Convention on Biological Diversity. This section provides that countries should "(a)dopt measures for the *ex-situ* conservation of components of biological diversity, **preferably in the country of origin** of such components" (emphasis added).

In the context of biodiversity, the costs and benefits of any particular arrangement must be balanced on an international scale. Article 11 of the Convention on Biological Diversity calls upon nations to implement incentive measures for the conservation and sustainable use of biodiversity, as exemplified by the United States' grants to multinational biodiversity development consortia. Such programs are intended to promote overall benefit, not just those within the United States. In a sense, as Gordon Cragg points out, such grants take on a dimension of foreign aid. Foreign aid is more acceptable in the case of a poor country, and less acceptable where it benefits the industry of a wealthy country, like Sandoz in Switzerland. The better approach is to try to structure market incentives for conservation, and to prime the pump and supplement where necessary with financial assistance.

2. What legal mechanisms exist whereby those who provide botanical samples can share benefits deriving from use of the samples?

The legal tools currently available to providers of genetic resources include sovereign rights, contracts, trade secret law and statutory intellectual property rights such as patent systems. These mechanisms may not be adequate in some situations, and so there is also pressure for novel types of intellectual property rights for indigenous knowledge.

Underlying the Convention on Biological Diversity is a shift away from the view that the genetic wealth of nations is a common heritage of humanity, to the view that states have sovereign rights over genetic resources, and may restrict access to them (Articles 3 and 15). A nation may impose conditions on access, such as a fee or royalty arrangement.

One exception is genetic material in existing gene banks. The Convention has a "grandfather clause" excluding such material from the access and technology transfer provisions [Article 15(3)]. How such rights will be allocated is currently under debate.

It is straightforward under contract law for someone who knows ethnobotanic or taxonomic information to provide that information to someone else for consideration. For example, a shaman can sell ethnobotanic information, and receive compensation even if the shaman is not an inventor under the law. However, once the information becomes public, it has no further value as a trade secret, and in any event, the shaman has no rights against a third party who obtains the information from public sources.

Contracting for trade secrets is thus not an adequate model for providing compensation. The parties to a contract may have vastly different bargaining power. An

individual may not have capacity to contract on behalf of a tribe. A statutory intellectual property right, good against all, is much more valuable and may be more effective at steering benefits to the people in the source country who are able to protect habitats.

Trade secrets are not well-protected by law in many countries. Therefore, shamans have evolved the practice of protecting their knowledge by, e.g., mixing an active ingredient with other substances, and shrouding the treatment in a cultural ritual. Ethnobotanists dating back to Nicole Maxwell in the 1950s have collected ethnobotanic information from indigenous people, in confidence, and then published it, thus effectively eliminating the possibility for any one in the tribe or in future generations to obtain compensation for disclosure of the information. There are many legal hurdles facing someone who would seek redress for such a disclosure.

A materials transfer agreement generally includes restrictions on the use of the sample being transferred, allocates rights in patents and other intellectual property that may derive from the sample, and may provide for confidentiality. Agreements such as the NCI Letter of Intent may provide compensation for source countries and people with botanical information, and can avoid the stringent substantive requirements and expensive prosecution necessary to obtain patent prosecution.

Statutory intellectual property rights (e.g., patents, copyrights, and trademarks) can reward innovation, provide incentives for disclosure, and facilitate trade and technology transfer. Consistent with the land ethic discussed above, it is desirable for a person with information useful to conserving biodiversity, such as ethnobotanic information, to have some kind of intellectual property right. This policy must be balanced with the need to provide adequate incentives for industrial companies developing useful products and methods from the starting material.

The key questions for any intellectual property system are (1) What can be protected? and (2) Who holds rights? and (3) How is protection obtained?

Patent protection has drawbacks as a way to support providers of genetic material and botanic information. Patent systems are national, and the trend towards patent harmonization is not assured. Administrative and legal costs are great, and the results uncertain. In many countries, living organisms and biotechnology inventions are not qualified for patent protection.

As for taxonomic information, protection is also available under copyright law for databases and publications. To some extent, as the discussion of screening in Madagascar points out, the notion of "random screening" is a fallacy. A collector always makes some selection based on botanical knowledge, and that information may be protectible.

There is no internationally recognized intellectual property right for long-standing traditional knowledge, although at least one country, Kenya, has begun to experiment with petty patent protection for traditional medicinal knowledge. We can expect to see further experiments in this area as countries seek ways to protect their biological and cultural diversity.

The United Nations Environment Programme is considering measures for using intellectual property to protect indigenous knowledge relevant to biodiversity. Unfortunately, no workable intellectual property system has yet been proposed. Perhaps the simpler approach of providing subsidies or monetary awards from funds allocated under the Convention on Biological Diversity to those with information about the bioactivity of a plant, fungus, or bacteria would be effective. The Healing Forest Conservancy is a private effort in the same direction.

3. When is a traditional user of a plant or a collector an inventor of a resulting drug or compound?

Mere discovery of a biologically active plant or extract would not generally be patentable. In considering who would be an inventor of a patentable invention deriving from biodiversity prospecting, we must first assume that there is indeed an invention.

The standard of joint inventorship is whether the person collaborated with others and contributed conceptually to the solution of a problem. They must be working toward a common end on the same subject matter, although they need not work physically in the same place or at the same time (35 U.S.C. 116). One who merely supplies background data or general information to an inventor does not become an inventor; this point was established in litigation over Morse's telegraph, where scientists who consulted with Morse were held not to be co-inventors [O'Reilly v. Morse, 56 U.S. (15 How.) 62 (1853)].

In the context of biodiversity, then, a shaman, ethnobotanist, or other person providing ethnobotanic information that is not patentable in and of itself would not be a joint inventor under U.S. law unless: (a) the information contributes to the conception of the claimed invention; (b) the information was obtained or applied in collaboration with the inventor who isolates or does further work on the compound that is patented: and (c) there is a conceptual connection between the information and the ultimate end use.

Element (c) may be particularly hard to satisfy, because the link between a preparation of a medicinal plant, the natural compound in a plant , a purified form of the compound, a synthetic form, a derivative, the gene for the compound, or a genetically engineered form of the compound can become quite tenuous. With random collection, there would be no connection. With a targeted strategy, there might be. Where the medicinal use of a plant has nothing to do with the patented use or compound, such as a tribal healer's snake bite cure being used to increase milk production, it is difficult to imagine how the person who recognized the medicinal use would be an inventor under U.S. law. If it can be shown that **but for** the ethnobotanical input, the invention would not have been made, as with a snake bite cure being used to produce a coagulant, then element (c) would likely be satisfied.

In conclusion, the recognition of proprietary rights over genetic resources raises complicated issues of policy and law. It will take much trial and error to come up with practical solutions that promote access to and development of wild and *ex situ* genetic resources while conserving biodiversity and assuring that benefits are equitably shared.

REFERENCES

Leopold, Aldo. 1966. "A Sand County Almanac." Oxford University Press.

Missouri Botanical Garden Library
3 1753 00204 3112

ISBN 0-915279-20-7